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13. ABSTRACT (Maximum 200 Words)

Most prostate cancer patients respond initially to androgen ablation and antiandrogen therapy. However, virtually all patients will relapse due to acquisition of the growth of the androgen-independent tumor cells. The molecular mechanism characterizing prostate cancer progression from androgen-dependence to androgen-independence is incompletely understood. We propose that Signal Transducers and Activators of Transcription 3 (Stat3) both regulates he expression of Stat3 target genes, and interacts with AR in prostate cancer cells. The experiments proposed in this application are based upon the hypothesis that Stat3 activation alters androgen receptor signaling pathways, that in turn results in the loss of growth control in prostate cancer cells. We propose to determine the consequence of Stat3 activation in prostate cancer cell growth and to determine the molecular basis of Stat3 interactions with androgen receptor signaling.

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Introduction

Most prostate cancer patients respond initially to androgen ablation and antiandrogen therapy. However, virtually all patients will relapse due to acquisition of the growth of the androgen-independent tumor cells. The molecular mechanism characterizing prostate cancer progression from androgen-dependence to androgen-independence is incompletely understood. We propose that Signal Transducers and Activators of Transcription 3 (Stat3) both regulates the expression of Stat3 target genes, and interacts with AR in prostate cancer cells. The experiments proposed in this application are based upon the hypothesis that Stat3 activation alters androgen receptor signaling pathways, that in turn results in the loss of growth control in prostate cancer cells. We propose to determine the consequence of Stat3 activation in prostate cancer cell growth and to determine the molecular basis of Stat3 interactions with androgen receptor signaling.

Body

Since the approval of this application, we have made significant progress of task 1 (i.e., to examine the role of Stat3 activation in prostate cancer cells (months 1-18).

Task 1A. To establish a series of prostate cancer cell lines demonstrating constitutive Stat3 activation (months 1-6). We have established Stat3 constitutively activated cell sublines in LNCaP cells. These cells express activated Stat3 as examined by EMSA using Stat3 consensus binding sequences (reference 1).

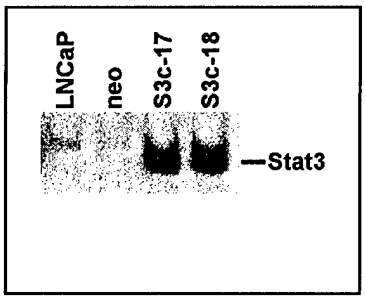
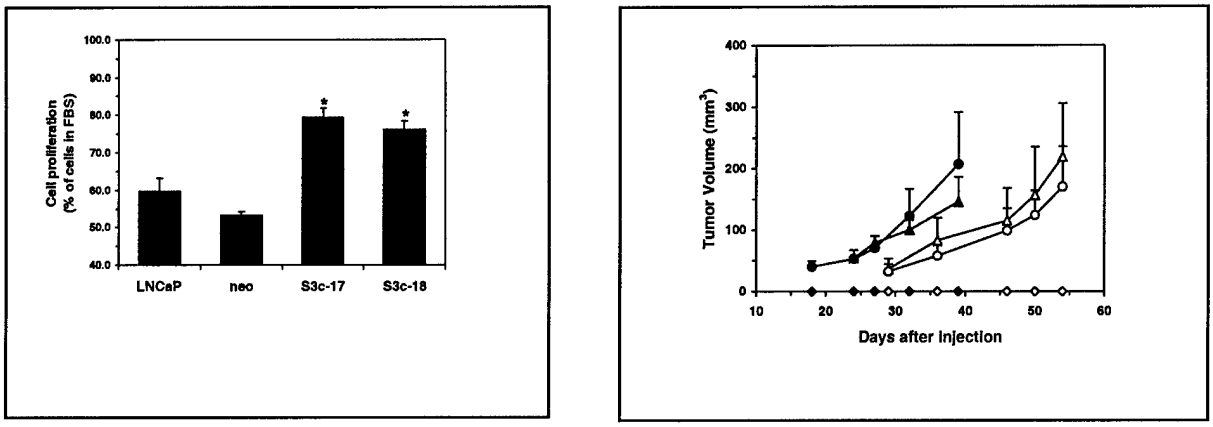


Fig 1. Stat3 DNA-binding activity in LNCaP and LN series cell lines. Whole cell extracts (20 μg) were subjected to EMSA using a ^{32}P-labeled oligonucleotide probe containing the consensus binding motif for Stat3.

Task 1B. To examine the effect of Stat3 activation on these cell growth in vitro and in vivo (months 7-12). We demonstrated that cells expressing constitutively activated Stat3 can enhance LNCaP androgen independent growth in vitro as analyzed by MTT assay (Fig 2A) and LNCaP growth in the castrated nude mice (Fig 2B), suggesting that Stat3 can enhance androgen independent growth of androgen-dependent LNCaP cells (reference 1).



A **B**

Figure 2. Stat3 enhances androgen-independent growth *in vitro*. **(A)** Effect of Stat3 on LNCaP cell growth in the presence and absence of androgen *in vitro*. Cells were cultured in RPMI-1640 supplemented with either 10% FBS or 10% charcoal-stripped FBS. Cell proliferation values in charcoal-stripped FBS were expressed as % relative to the complete FBS. *, $P < 0.05$. **(B)** Stat3 induces androgen-independent growth *in vivo*. Tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone (◇) or clones that overexpress activated Stat3 (S3c-17, ∇; S3c-18, O) were injected into the intact (filled symbols) or castrated (open symbols) male nude mice (n = 10 for each condition).

Task 1C. To examine the effect of Stat3 activation on the expression of Stat3 target genes and AR-mediated genes (months 7-18). Prostate specific antigen (PSA) is a typical AR-mediated gene. We demonstrated that Stat3 can enhance PSA express both in mRNA levels as examined by Northern blot (Fig 3A) and in protein levels as examined by ELISA (Fig 3B). In addition, Stat3 can enhance PSA promoter activity and ARE-containing gene transactivation (Fig 4 and reference 1).

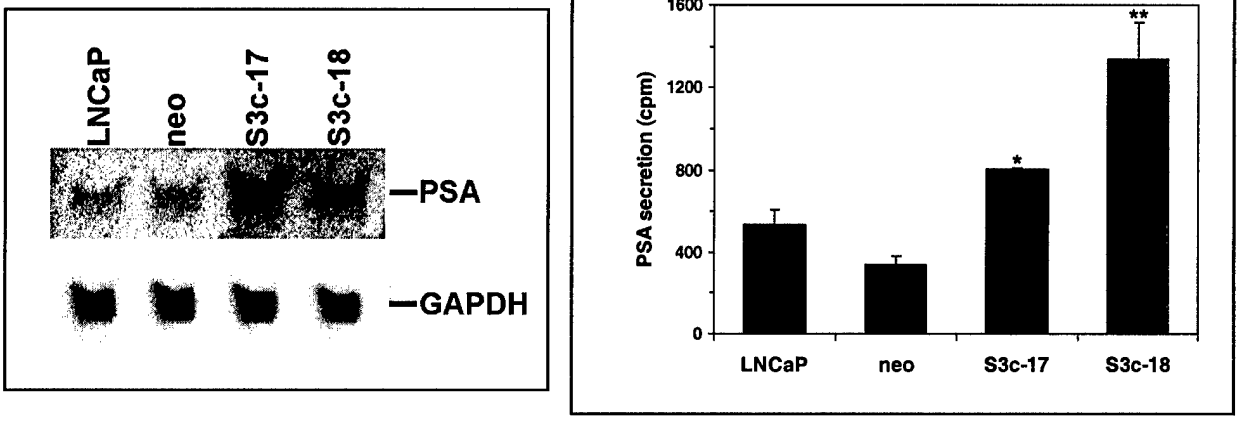


Figure 3. Stat3 enhances PSA expression. (A) PSA mRNA expression in Stat3 overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by Northern blot analysis using 20 μg of total RNA. GAPDH is a control for equal loading. (B) PSA protein secretion in the absence of androgen. PSA secretion was quantitated by PSA immunoradiometric assay of 50 μl of supernatant of cell culture in phenol red-free RPMI containing 10% charcoal-stripped serum. *, $P < 0.05$; **, $P < 0.01$.

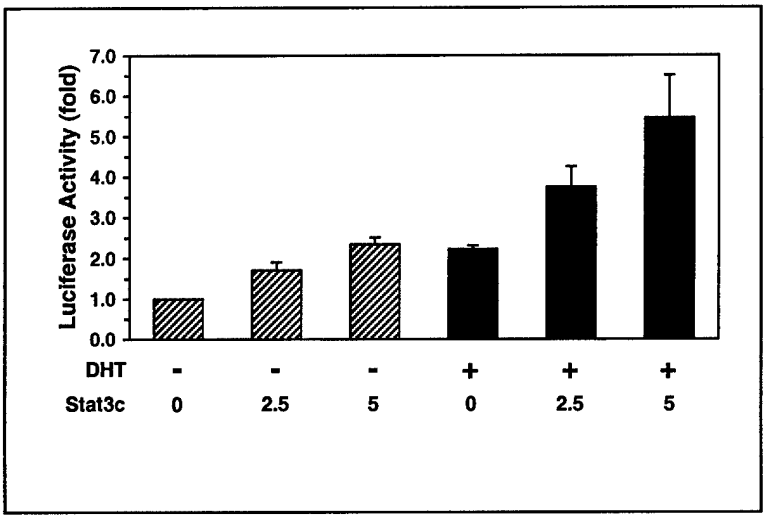


Figure 4. (A) Effect of Stat3 on PSA promoter activity in the absence of DHT and in the presence of 10 nM of DHT. LNCaP cells were transiently transfected with PSA-luc reporter, and increasing doses (0, 2.5, 5 μg) of Stat3 expression plasmid. Total DNA content was kept constant in all wells. HeLa cells transiently transfected with or without AR expression plasmid, PSA-luc reporter, and increasing doses (0, 2.5, 5 μg) of Stat3c expression plasmid in the presence of 10 nM of DHT. Total DNA content was kept constant in all wells. The luciferase activity was measured. Results are displayed as the average of four independent experiments. RLU: relative light units.

Task 2. To determine whether Stat3 activation induce androgen-independent growth by affecting AR signaling in prostate cancer cells (months 19-36).

We are initiating the experiments to study the interaction between Stat3 and AR and the consequences of this interaction in promoting androgen-independent prostate cancer. The experiments were designed to investigate the potential use of RNA interference to block Stat3 expression and activation and the effect on the growth of human prostate cancer cells. We identified a small interfering RNA (siRNA) specific for Stat3 and expressed in human prostate cancer cells from DNA expression vector which employ RNA polymerase III promoters from the U6 small nuclear RNA gene to transcribe siRNAs. We demonstrate that blockade of Stat3 activation by the Stat3 siRNA suppresses the growth of human prostate cancer cells and Stat3-mediated gene expression and induces apoptotic cell death. The Stat3 siRNA does not inhibit the proliferation nor induces apoptosis of Stat3-inactive human prostate cancer cells. In addition, the Stat3

siRNA inhibits the levels of AR-mediated gene- prostate specific antigen (PSA) expression in prostate cancer cells.

Androgen ablation induces apoptotic death of prostate epithelial cells and is a standard treatment for prostate cancer. However, androgen-independent prostate cancer cells become resistant to apoptosis, rendering androgen ablation therapy ineffective. To understand the role of Stat3 in androgen independent prostate cancer, we investigated the role of Stat3 activation in IL-6-mediated antiapoptotic activity in prostate cancer cells. We demonstrate that overexpression of IL-6 renders androgen sensitive LNCaP human prostate cancer cells more resistant to apoptosis induced by androgen deprivation. LNCaP cells undergo apoptosis after 72 h of androgen deprivation, an outcome is largely absent in clones overexpressing IL-6 as measured by cell death ELISA and chromatin degradation assays. IL-6 over-expressing cells resulted in a significant decrease in the expression of pro-apoptotic proteins such as cleaved PARP and cleaved caspase-9 as well as an increase in the expression of anti-apoptotic proteins Bcl-x_L and phosphorylated Bad. Addition of IL-6 antibody completely abolished the anti-apoptotic activity of IL-6. This protective effect of IL-6 was reversed by the expression of a dominant-negative Stat3 mutant, Stat3F. Furthermore, ectopic expression of a constitutively active Stat3 antagonized androgen deprivation-induced cell death of LNCaP cells. These results indicate that IL-6 protects androgen sensitive LNCaP cells from apoptosis induced by androgen deprivation, and Stat3 activation play an important role in IL-6-mediated anti-apoptosis in prostate cancer cells.

Key research accomplishments

- We demonstrated that Stat3 plays a critical role in prostate cancer growth.
- Stat3 enhances AR-mediated gene expression such as PSA.
- Stat3 enhances androgen independent growth of prostate cancer cells *in vitro* and *in vivo*.
- Stat3 activates androgen receptor (AR) in the presence and in the absence of androgen.
- Stat3 activation is required for IL-6 mediated antiapoptic activity in prostate cancer cells.
- Targeting Stat3 signaling by siRNA inhibits growth and induces apoptosis of prostate cancer cells.

Reportable outcome

Manuscripts:

1. Lee SO, Lou W, Demiguel F, Hou M, **Gao AC.** Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. Clin. Cancer Res., 9: 370-376, 2003.
2. Lee S.O., Lou W., Hou, M., Onate S.A., and **Gao A.C.** Interleukin-4 enhances prostate specific antigen expression by activation of the androgen receptor and Akt pathway. Oncogene, 2003 (in press)

3. Demiguel F, Lee S.O., Onate A. A., and **Gao AC.** Stat3 enhances transactivation of steroid hormone receptors. BioMed Central, Nuclear Receptor, 1:3, 2003.

4. Leman ES, DeMIGUEL F, **Gao AC**, Getzenberg RH. Regulation of Androgen and Vitamin D Receptors by 1,25-Dihydroxyvitamin D3 in Human Prostate Epithelial and Stromal Cells. J Urol. 170: 235-240, 2003.

5. Wei D, Le X, Zheng L, Wang L, Frey JA, **Gao AC**, Peng Z, Huang S, Xiong HQ, Abbruzzese JL, Xie K. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. Oncogene 23;22(3):319-29, 2003.

6. DeMigeul F., Lou W., Lee S. O., Xiao X., Pflug B., Nelson J.B., and **Gao A.C.** Stat3 enhances the growth of LNCaP human prostate cancer cells in intact and castrated male nude mice. The Prostate, 52: 1-7, 2002.

7. Soo Ok Lee, Wei Lou, Khusroo M. Qureshi, Farideh Mehraein-Ghomi, and **Allen C. Gao**. RNA interference targeting Stat3 inhibits growth and induces apoptosis of human prostate cancer cells. (submitted).

8. Soo Ok Lee, Wei Lou, Min Hou, Candace S. Johnson, Donald L. Trump and **Allen C. Gao**. Interleukin-6 protects LNCaP cells from apoptosis induced by androgen deprivation through the Stat3 pathway. (submitted).

Conclusions

We demonstrated that activation of Stat3 in androgen-sensitive LNCaP prostate cancer cells results in enhancement of tumor growth in both intact and castrated male nude mice, and enhances androgen receptor-mediated prostate specific antigen (PSA) expression. These findings demonstrate that intracellular signaling mediated by Stat3 can enhance the growth of androgen-sensitive human LNCaP prostate cancer cells in both intact and castrated male nude mice. Targeting Stat3 signaling by siRNA may serve as a novel therapeutic approach for prostate cancer.

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9. DeMigeul F., Lou W., Lee S. O., Xiao X., Pflug B., Nelson J.B., and **Gao A.C.** Stat3 enhances the growth of LNCaP human prostate cancer cells in intact and castrated male nude mice. The Prostate, 52: 1-7, 2002.
10. Lee S.O., Lou, W., Demiguel F, Hou, M., and Gao, A.C. Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. Clin Cancer Res. (in press).

Interleukin-6 Promotes Androgen-independent Growth in LNCaP Human Prostate Cancer Cells[1]

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ABSTRACT

Purpose: **Prostate cancer frequently progresses from an initial androgen dependence to androgen independence, rendering the only effective androgen ablation therapy useless. The mechanism underlying the androgen-independent progression is incompletely understood. Interleukin (IL)-6 has been implicated in this androgen-independent progression. In this study, we tested whether IL-6 induces androgen-independent growth both *in vitro* and *in vivo*.**

Experimental Design: **IL-6 was expressed in androgen-sensitive LNCaP cells. The effects of IL-6 on androgen receptor activity was determined by Northern blots and gel shift assays. The effects of IL-6 on LNCaP cell growth were determined *in vitro* by MTT assay and *in vivo*.**

Results: **IL-6 can enhance the growth of androgen-sensitive LNCaP cells in the androgen-deprived condition *in vitro*, which is accompanied by elevation of androgen-regulated prostate-specific antigen mRNA expression. IL-6 promotes androgen-sensitive LNCaP cell tumor growth in the castrated male mice. IL-6 enhances androgen receptor DNA binding activity and nuclear translocation. The androgen-independent phenotype induced by IL-6 in LNCaP cells is accompanied by significant activation of signal transducers and activators of transcription 3 and mitogen-activated protein kinase signal pathways.**

Conclusions: **These studies clearly provide experimental evidence that IL-6 initiates and/or enhances the transition of prostate cancer cells from an androgen-dependent to an androgen-independent phenotype.**

INTRODUCTION

The growth of prostate epithelial cells requires a physiological level of androgen, both to stimulate proliferation and inhibit apoptotic death (1). Androgen binds to the AR,[4] which triggers interaction of AR to specific AREs in the promoters of androgen-regulated genes. These interactions facilitate the activation or repression of genes regulating development, differentiation, and proliferation of prostate epithelial cells. Currently, the standard treatment for metastatic prostate cancer is androgen ablation therapy. The problem is that whereas almost all patients with prostate cancer initially respond to androgen ablation therapy, virtually every patient will relapse to hormone-refractory disease due to the growth of androgen-independent cancer cells, rendering the only effective therapy useless. The molecular cause of acquired androgen-independent growth, which is promoted by activation of AR signaling through AR gene mutation and amplification (2, 3), coactivators (4), and cross-talk between the AR and protein kinase pathways (4, 5), is incompletely understood. There is growing evidence that suggests growth factors and cytokines play an important role in acquisition of hormone independence.

IL-6 is a glycoprotein consisting of 212 amino acids encoded by the IL-6 gene localized to chromosome 7p21–14 (6). IL-6 is a pleiotropic cytokine that plays a central role in host defense mechanisms by regulating immune responses, hematopoiesis, and the induction of acute phase reaction (6). The biological activities of IL-6 are mediated by the IL-6 receptor. The receptor for the IL-6 family of cytokines (IL-6, IL-11, ciliary neurotrophic factor, oncostatin M, and leukemia inhibitory factor) is composed of an IL-6-specific receptor subunit (α chain) and a signal transducer, gp130 [β chain (7)]. The binding of IL-6 to its receptor resulted in activation of intracellular signaling including Janus kinase-Stat and MAPK pathways (7, 8).

The expression and function of IL-6 in prostate cancer have been the subject of multiple recent studies. The expression of IL-6 and its receptor has been consistently demonstrated not only in human prostate cancer cell lines but more importantly in human prostate carcinoma and benign prostate hyperplasia obtained directly from patients (9–11). The levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer (12, 13). Furthermore, IL-6

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[4] The abbreviations used are: IL, interleukin; AR, androgen receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PSA, prostate-specific antigen; Stat, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; ARE, androgen-responsive element; FBS, fetal bovine serum; CS, charcoal-stripped; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift analysis; PI3K, phosphatidylinositol 3′-kinase; ERK, extracellular signal-regulated kinase.

has been demonstrated as a candidate mediator of human prostate cancer morbidity (14). IL-6 has been suggested to have both growth-promoting and -inhibiting activities in androgen-dependent LNCaP human prostate cancer cells *in vitro*. IL-6 can function as a paracrine growth factor for the human LNCaP androgen-sensitive prostate cancer cells and an autocrine growth factor for human DU145 and PC3 androgen-insensitive prostate cancer cells (15–18). IL-6 can also function as a paracrine growth inhibitor for LNCaP cells and an autocrine growth stimulator for the DU145 and PC3 cells (19). Recently, results from a number of groups demonstrated that IL-6 activates AR-mediated gene expression in LNCaP cells *in vitro* (17, 20–22), suggesting that IL-6 may play a critical role during the progression of prostate cancer.

Whereas numerous studies have suggested the role of IL-6 in the growth and androgen responsiveness of prostate cancer cells *in vitro*, there is no experimental evidence to demonstrate the role of IL-6 in the promotion of androgen-independent growth of prostate cancer cells *in vivo*. In this study, we tested whether IL-6 induces androgen-independent growth. We demonstrate that IL-6 induces androgen-independent growth of androgen-sensitive LNCaP human prostate cancer cells both *in vitro* and *in vivo*, which is accompanied by elevation of PSA levels. The androgen-independent phenotype induced by IL-6 in LNCaP cells is mediated in large part by activation of Stat3 signaling and potentially also by activation of the MAPK pathway.

MATERIALS AND METHODS

Cell Culture. The LNCaP cells were maintained in RPMI 1640 containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FBS at 37°C in 5% CO_2 incubator. The IL-6-overexpressing cells (LN-S15 and LN-S17) and neo control (transfected with vector alone) cells were cultured in the same medium plus 0.3 mg/ml G418. To investigate the androgen withdrawal effect, cells were cultured in medium containing 10% CS-FBS instead of regular 10% FBS.

***In Vitro* Cell Proliferation.** LNCaP cells or IL-6-overexpressing cells (LN-S15 and LN-S17; 10^4 cells/well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 2 or 3 days in regular culture medium with 10% FBS, cells were switched into a medium of phenol red-free RPMI 1640 containing either 10% FBS or 10% CS-FBS (Hyclone). For controls, antihuman IL-6 antibody (20 μg/ml; Sigma) was added into the tissue culture medium. Two days later, cells were determined by using the MTT assay (Sigma) according to the manufacturer's instructions.

***In Vivo* Tumor Growth.** Four- to six-week-old athymic male nude mice (Harlan, Indianapolis, IN) were inoculated s.c. in the flank with 3×10^6 cells (LNCaP, neo, LN-S15, and LN-S17) resuspended in Matrigel (BD Biosciences, Bedford, MA) diluted 1:1 in complete culture medium. The volume of the growing tumors was estimated by measuring three tumor dimensions (length × width × depth) with a caliper (23).

RT-PCR. RT-PCR was performed as follows. Briefly, total RNA was isolated from cells using the Trizol method (Life Technologies, Inc., Rockville, MD). One μg of total RNA was used in the reverse transcription reaction, and thermal cycling was programmed as follows: 1 min at 4°C; 2 min at 70°C; and 5 min at 4°C with oligodeoxythymidylic acid. After chilling tubes on ice, buffer, deoxynucleotide triphosphates, RNase inhibitor, and mouse mammary tumor virus were added and incubated at 42°C for 1 h. The cDNAs thus obtained were amplified with 30 cycles (45 s at 95°C, 1 min at 58°C, and 1 min at 72°C) of PCR reaction in the presence of Taq polymerase (Promega, Madison, WI). PSA primer sequences used were 5′-GGCAGGTGCTTGTAGCCTCTC-3′ (sense) and 5′-CACCCGAGCAGGTGCTTTTGC-3′ (antisense). The PCR products were then resolved in a 1.5% agarose gel, and bands were analyzed with Molecular Imager FX System (Bio-Rad, Hercules, CA). GAPDH primers were used as control.

Northern Blot. Twenty μg of RNAs were electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 1.1-kb *Bam*HI fragment containing the PSA cDNA was labeled with [α-^{32}P]dCTP (3000 Ci/mmol; ICN, Costa Mesa, CA) using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Hybridization was carried out during 3 h at 65°C in Rapid-hyb buffer (Amersham Pharmacia Biotech). Membranes were washed for 15 min at 65°C in 2× SSC, 0.1% SDS (twice); 0.5× SSC, 0.1% SDS; and 0.1× SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio-Rad).

Determination of PSA Secretion. The serum was collected at the end of experiments. Fifty μl of serum were used to determine PSA secretion. Levels of PSA in the serum of tumor-bearing mice were determined by ELISA with the use of anti-PSA as primary antibody as described by the manufacturer's protocol (Bechman Coulter, Fullerton, CA).

EMSA. Whole cell extracts were prepared by using high-salt buffer [20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM $Na_2P_2O_7$, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 420 mM NaCl, 20% glycerol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin], followed by snap-freezing in ethanol/dry ice for 5 min and thawing on ice for 10 min. The freeze and thaw procedures were repeated again for a total of two times. The supernatant was then centrifuged and harvested. Protein concentrations were determined by Coomassie Blue plus protein assay kit (Pierce) according to the manufacturer's protocol. Stat3 DNA binding activity was determined by EMSA using Stat3 consensus oligonucleotide 5′-GATCCTTCTGGGAATTCCTAGATC as described previously (24). For determination of the AR DNA binding activity, whole cell extracts (20 μg) were incubated in a final volume of 20 μl [10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μg/ml poly(deoxyinosinic-deoxycytidylic acid)] by EMSA with radiolabeled double-stranded AR consensus binding motif (Santa Cruz Biotechnologies, Santa Cruz, CA). The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25× Tris-borate EDTA at room temperature, and the results were autoradiographed. Quantitation of the amount of AR DNA binding activity in the "protein-DNA" bandshift was measured using the Molecular Imager FX System (Bio-Rad). For the supershift experiment, 20 μg of cell extracts were incubated with either Stat3 antibody or AR antibody

(Santa Cruz Biotechnologies) for 1 h at 4°C before incubation with the radiolabeled probe.

Nuclear Lysate Preparation. Nuclear protein extracts were prepared as described previously (17). Briefly, for nuclei preparation, cells were harvested, washed with PBS twice, resuspended in hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% NP40], and incubated on ice for 10 min. Nuclei were precipitated with 3,000 × *g* centrifugation at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100] and incubated on ice for 30 min. The nuclear lysates were precleared by 20,000 × *g* centrifugation at 4°C for 15 min. Protein concentration was determined by Coomassie Blue plus protein assay kit.

Western Blot Analysis. Forty μg of protein were resolved in 8–12% SDS-PAGE, depending on the molecular weight of the protein to be detected. After blocking overnight at 4°C in 5% milk in PBS-0.1% Tween 20, membranes were incubated overnight with antibodies against either Stat3, phosphorylated Stat3, p44/42ERK1/2, phosphorylated p44/42ERK1/2, Akt, phosphorylated Akt (Cell Signaling Technology) or AR (Santa Cruz Biotechnology). After secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS

Effect of IL-6 on the Growth of LNCaP Cells *in Vitro*. It was demonstrated previously that the androgen-sensitive LNCaP cells express IL-6 receptor but express no detectable IL-6 protein (15–19). IL-6 can enhance AR-mediated PSA expression in LNCaP cells (17, 18, 20–22), suggesting that IL-6 can enhance androgen responsiveness of LNCaP cells. To determine the effect of IL-6 on the growth of LNCaP cells in the presence and absence of androgen, we ectopically expressed IL-6 by introduction of a full-length IL-6 cDNA into IL-6-negative LNCaP cells as described previously (16). Several stable transfectants containing IL-6 cDNA in the sense orientation and vector-alone controls were selected in the presence of G418, subcloned, and tested for their expression of IL-6 by ELISAs. Two stable IL-6 transfectants (LN-S15 and LN-S17) expressing high levels of IL-6 (2465 and 2743 pg/ml/10^6 cells, respectively) were selected for additional studies. To test whether IL-6 promotes LNCaP androgen-independent cell growth *in vitro*, parental LNCaP, neo control, and IL-6-overexpressing clones were cultured in the presence and absence of androgen, and the cell growth was determined. As shown in Fig. 1*A*, the growth of androgen-sensitive LNCaP cells and neo control in culture was reduced by about 50% after 48 h in androgen-deprived CS serum (Hyclone; testosterone concentration is $<10^{-11}$ M, in which prostate epithelial cells do not respond to testosterone stimulation; Ref. 25) compared with that in normal serum (testosterone concentration is about 10^{-9} M). Addition of dihydrotestosterone (10^{-9} M) in the CS serum restored LNCaP cell growth to levels similar to that of the complete normal serum (data not shown). In the clones of LNCaP cells overexpressing IL-6 (LN-15 and LN-17), however, there was only a 5–10% decrease in growth under these androgen-deprived conditions compared with growth in normal serum, suggesting that overexpression of IL-6 can enhance the growth of LNCaP cells in the androgen-deprived condition *in vitro*. Addition of anti-IL-6 antibody in IL-6 overexpression clones restored the growth inhibition to about 60% under androgen-deprived conditions compared with growth in normal serum.

IL-6 Induces Androgen-independent Growth *in Vivo*. Having demonstrated that IL-6 enhances the growth of androgen-sensitive LNCaP cells in the absence of androgen *in vitro*, we further tested the effect of overexpression of IL-6 on the growth of androgen-sensitive LNCaP human prostate cancer cells *in vivo*. To test the effects of IL-6 on tumor formation and induction of androgen-independent growth of LNCaP cells *in vivo*, 8-week-old male nude mice were randomly divided into two groups; one group was left intact, and the other group received surgical castration, in which the residual levels of androgen are insufficient to maintain growth of androgen-sensitive LNCaP cells (1). Three days after castration, intact or castrated male nude mice were inoculated s.c. with parental LNCaP, vector control neo, or LNCaP cell clones overexpressing IL-6 with coinoculation of Matrigel. For the two independent IL-6-overexpressing clones, tumors became apparent at the site of injection within 30 days in the intact male mice and within 40 days in the castrated male mice (Fig. 1, *B* and *C*). Parental LNCaP cells and vector control neo clone did not grow any detectable tumor in both intact (within a 40-day observation period) and castrated (within a 70-day observation period) male nude mice. These results demonstrate that IL-6 promotes the growth of androgen-sensitive LNCaP cells in the absence of androgen *in vivo*.

IL-6 Enhances Androgen-responsive Gene PSA Expression *in Vitro* and *in Vivo*. Results from a number of groups demonstrated that IL-6 activates AR-mediated PSA gene expression in LNCaP cells *in vitro* (17, 20–22). To test whether overexpression of IL-6 enhances the expression of an endogenous, androgen-regulated PSA, the expression of PSA was compared between the parental and IL-6-overexpressing LNCaP cells in the presence and absence of androgen. As shown in the Fig. 2*A*, in the presence of androgen, PSA mRNA expression was elevated in the IL-6-overexpressing LNCaP cells compared with the parental and vector control LNCaP cells. When the cells were cultured in phenol red-free medium supplemented with the CS serum, in which the androgen was deprived, PSA mRNA expression was elevated in the IL-6-overexpressing clones compared with the parental and vector control LNCaP cells (Fig. 2*B*), suggesting that overexpression of IL-6 can enhance endogenous PSA expression in the presence and absence of androgen. These results are consistent with previous reports that IL-6 activates the PSA promoter/enhancer in the presence and absence of androgen (17, 20, 21). In addition, tumors generated from IL-6-overexpressing LNCaP cells also produced high levels of circulating PSA in the serum of both the intact male mice (average, 38 ng/ml per g of tumor) and the castrated male mice (average, 32 ng/ml per g of tumor).

To test whether IL-6 can influence the DNA binding activity of AR protein to the ARE, we performed EMSA using radiolabeled oligonucleotides of the ARE with nuclear extracts from LNCaP cell clones. LNCaP clones overexpressing IL-6

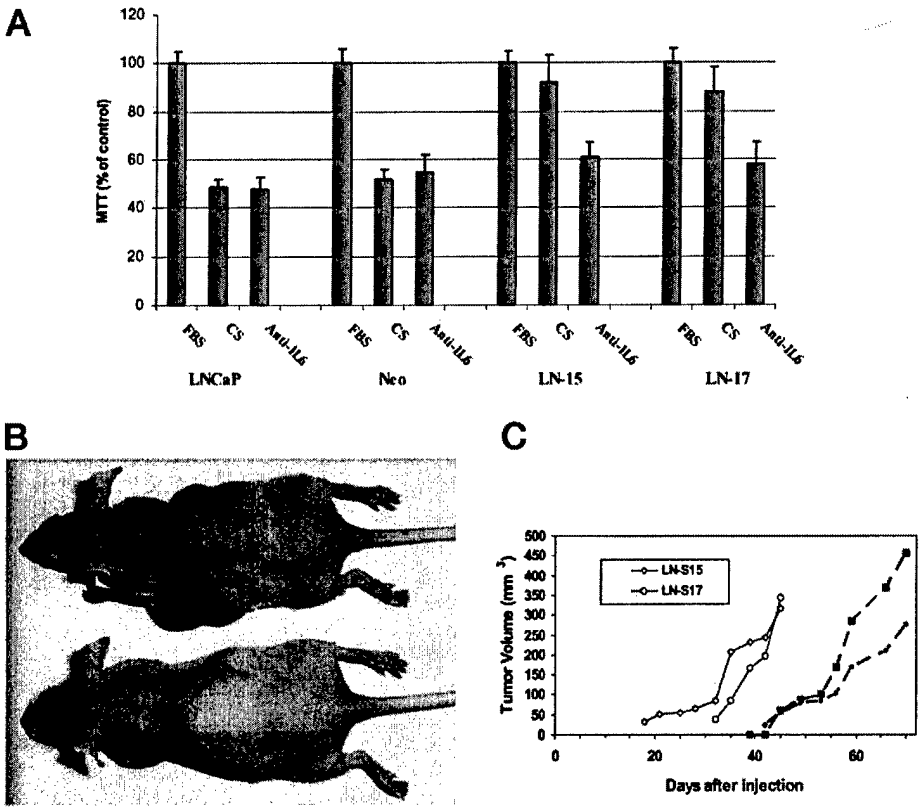

Fig. 1 Overexpression of IL-6 promotes LNCaP androgen-independent growth. *A*, effect of overexpression of IL-6 on LNCaP cell growth in the presence and absence of androgen *in vitro*. Cells were cultured in RPMI 1640 supplemented with 10% FBS. After 24 h, the cells were switched to either 10% FBS or 10% CS-FBS (*CS*). For controls, cells were cultured in RPMI 1640 supplemented with 10% CS-FBS plus 20 μg/ml anti-IL-6 antibody. After incubation for another 72 h, MTT assays were performed. MTT values for the complete FBS were expressed as 100%, and MTT values for CS-FBS were expressed as a percentage relative to complete FBS. *B*, IL-6-overexpressing clone (LN-15) developed tumors *versus* parental LNCaP cells that did not grow any tumor in the castrated male nude mice. *C*, tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone or IL-6-expressing clones (LN-15 and LN-17) were injected into the intact (*solid lines, open symbols*) or castrated (*broken lines, filled symbols*) male nude mice (n = 8 for each condition). Parental LNCaP and neo control did not grow tumor.

showed an increase in AR-ARE complex formation compared with the parental LNCaP cells in the presence of androgen as well as in the absence of androgen (Fig. 3*A*). The AR-ARE complex in the IL-6-overexpressing clones has little change in the absence of androgen compared with that in the presence of androgen (Fig. 3*A*). The specificity of this AR-ARE complex was demonstrated by supershift assay using antibody specifically against AR (Fig. 3*B*).

The AR typically translocates to the nucleus to exert its function on gene expression. To examine whether overexpression of IL-6 affects the expression and translocation of AR, Western blot analysis was performed using cell extracts from either whole cell extracts or nuclear extracts. As shown in Fig. 3*C*, overexpression of IL-6 in LNCaP cells significantly enhanced the expression of AR in the nuclear compartment without alteration of the total AR expression (whole cell extracts) both in the presence of androgen (FBS) and in the absence of androgen (CS-FBS).

Overexpression of IL-6 Activates Its Downstream Signaling Pathways in LNCaP Cells. The effects of IL-6 on prostate cancer cells are mediated by a variety of signal transduction pathways including Janus kinase-Stat, MAPK, and PI3K-AKT pathways, resulting in proliferation, differentiation, and inhibition of apoptosis. To examine which pathways were altered by overexpression of IL-6 in LNCaP cells, cell lysis from parental and IL-6-overexpressing LNCaP cells were analyzed. We first examined the effect of overexpression of IL-6 on the expression and activation of Stat3, a major mediator of IL-6 signaling. As shown in Fig. 4*A* and 4*B*, overexpression of IL-6 significantly elevates the activity of Stat3 both in the presence of androgen (FBS) and in the absence of androgen (CS-FBS).

To determine whether the increased Stat3 activity is associated with increased Stat3 protein expression and elevated phosphorylation, Western blots of whole cell extracts from the parental LNCaP and IL-6-overexpressing clones were performed using antibodies specific against either phosphotyrosine Stat3 (Tyr-705) or total Stat3 protein. As shown in Fig. 4*C*, overexpression of IL-6 induces Stat3 phosphorylation in LNCaP cells without alteration of total Stat3 expression, which is consistent with the results shown that IL-6 induces Stat3 activation in LNCaP cells. Collectively, these results demonstrate that

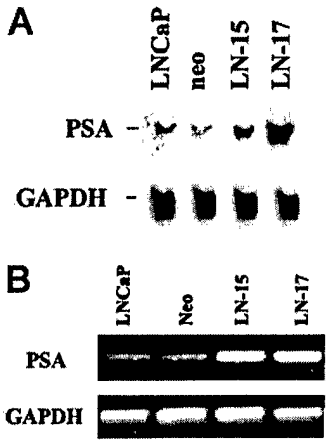

Fig. 2 Overexpression of IL-6 increases PSA mRNA expression in LNCaP cells. *A*, PSA mRNA expression in IL-6-overexpressing clones (LN-15 and LN-17), vector control (neo), and LNCaP cells cultured in normal FBS media and examined by Northern blot analysis using 20 μg of total RNA. GAPDH is a control for equal loading. *B*, PSA mRNA expression in IL-6-overexpression clones (LN-15 and LN17), vector control (neo), and LNCaP cells cultured in androgen-deprived CS-FBS media for 72 h. The PSA mRNA was examined by RT-PCR. GAPDH is a control for equal loading.

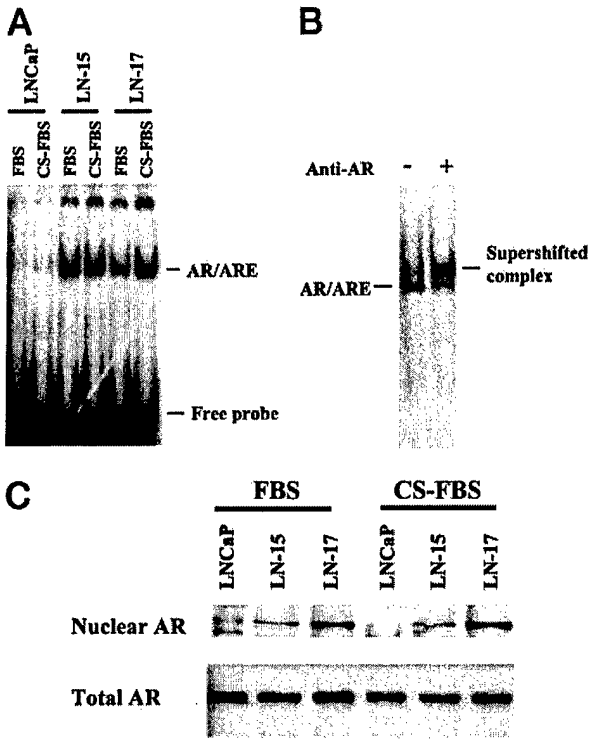

Fig. 3 Effect of overexpression of IL-6 on AR. *A*, IL-6 enhances the formation of AR-ARE complexes. EMSA was performed using radiolabeled ARE oligonucleotides with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. *B*, supershift assay of LN-17 cell extract using anti-AR antibody. Whole cell extracts were preincubated with antibodies specifically against AR as indicated. The positions of the AR-ARE and the supershifted complexes were indicated. *C*, overexpression of IL-6 enhances nuclear AR expression in LNCaP cells in the presence of androgen (FBS), but not in the absence of androgen (CS-FBS). Total cellular extracts and nuclear extracts were subjected to Western blot analysis (40 μg/lane) using an antihuman AR antibody. The cells were cultured in RPMI 1640 with 10% FBS for 24 h and then switched to either 10% FBS or 10% CS-FBS, and culture continued for another 72 h. Cell lysis were extracted and used for the assays.

overexpression of IL-6 significantly elevates Stat3 signaling in androgen-dependent human LNCaP prostate cancer cells.

To investigate whether overexpression of IL-6 alters Akt or MAPK signaling pathways in LNCaP cells, we performed Western blot analysis on cell extracts from parental LNCaP and IL-6-overexpressing clones using antibodies that specifically recognize either phosphorylated Akt or phosphorylated MAPK (p44/42ERK1/2), respectively. As shown in Fig. 4*D*, overexpression of IL-6 in LNCaP cells enhances the levels of phosphorylated (active) p44/42 ERK1/2 expression without altering the expression of total p44/42 ERK1/2 in both the presence and absence of androgen, whereas overexpression of IL-6 in LNCaP cells has less effect on the expression of phosphorylated Akt or total Akt in both the presence and absence of androgen (Fig. 4*D*).

Collectively, these results indicate that IL-6-induced signaling in LNCaP cells is mediated primarily through Stat3 and MAPK signaling pathways.

DISCUSSION

In the present study, we provide experimental evidence that IL-6 plays an important role in the induction of androgen-independent growth of human prostate cancer cells. We demonstrate that overexpression of IL-6 in androgen-sensitive human LNCaP prostate cancer cells results in the conversion of androgen-independent growth of LNCaP cells both *in vitro* and *in vivo*. Overexpression of IL-6 also enhances endogenous PSA expression in LNCaP cells, consistent with previous reports that IL-6 increases AR-mediated gene activation (17, 20–22). In addition, we demonstrate that IL-6 signaling is primarily mediated through activation of the Stat3 and MAPK signaling pathways in LNCaP cells.

The potential role of IL-6 in the development and progression of prostate cancer cells has been suggested by numerous studies. Clinically, the levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer (12, 13). In addition, increased expression of IL-6 and IL-6 receptor has been demonstrated in prostate cancer tissues, and increased IL-6 receptor is correlated with increased proliferation of prostate cancer cells (9–11, 18). Experimentally, IL-6 has been suggested to have both growth-promoting and -inhibiting activities in androgen-dependent LNCaP human prostate cancer cells *in vitro* (16, 18, 19, 22). It has been demonstrated that IL-6 can act as a growth factor for both normal primary prostate epithelial cells and LNCaP prostate cancer cells *in vitro* (16–18). IL-6 stimulates prostate-specific protein expression in prostate carcinoma cells by activation of the AR and can be blocked by the antiandrogen bicalcutamide (17, 20–22), consistent with our finding that overexpression of IL-6 enhances endogenous PSA expression in LNCaP cells. It

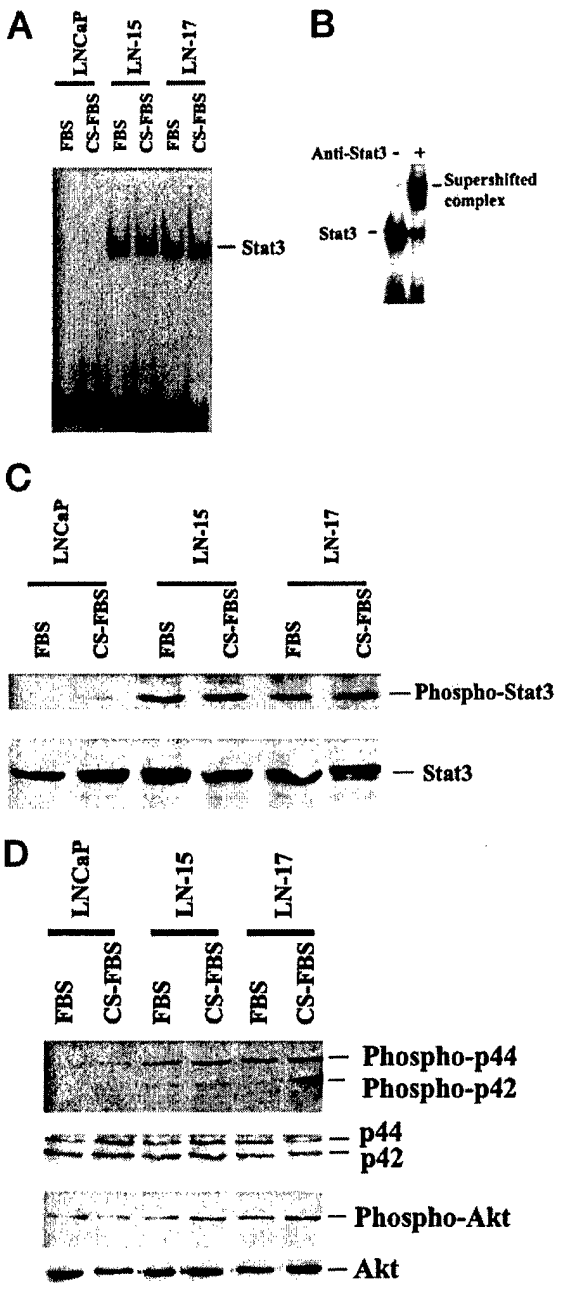

Fig. 4 Overexpression of IL-6 activates the Stat3 and MAPK pathways. *A*, IL-6 induces Stat3 activation in LNCaP cells. EMSA was performed using radiolabeled Stat3 oligonucleotides with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. *B*, supershift assay of LN-17 cell extract using anti-Stat3 antibody. Whole cell extracts were preincubated with antibodies specifically against Stat3 as indicated. The positions of Stat3 and the supershifted complexes were indicated. *C*, overexpression of IL-6 enhances Stat3 phosphorylation in LNCaP cells. Western blots were performed using antibodies against either phospho-specific Stat3 (Tyr-705) or total Stat3 with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. *D*, the effect of overexpression of IL-6 on Akt and MAPK expression in LNCaP cells. Whole cell extracts from parental LNCaP cells and IL-6-overexpressing clones (LN-15 and LN-17) cultured in either normal FBS or CS-FBS conditions were subjected to Western blot analysis using antibody against phospho-specific p44/42 ERK1/2 and reprobed with total p44/42 ERK1/2 or antibody against phospho-specific Akt (p-Akt Ser473) and reprobed with total Akt. The cells were cultured in RPMI 1640 with 10% FBS for 24 h and switched to either 10% FBS or 10% CS-FBS, and culture continued for another 72 h. Cell lysis were extracted and used for the assays.

has also been indicated that IL-6 can mediate LNCaP cell growth arrest and induction of neuroendocrine differentiation (26, 27). Whereas all of the observed effects of IL-6 on the growth of prostate cancer cells were performed in tissue culture cells, mostly in androgen-dependent LNCaP human prostate cancer cells *in vitro*, the potential effects of IL-6 on LNCaP cells *in vivo* have not been reported. The present study is the first to provide such experimental evidence that IL-6 induces androgen-independent growth of androgen-sensitive human LNCaP prostate cancer cells both *in vitro* and *in vivo*. We have observed that overexpression of IL-6 in LNCaP cells significantly activates the Stat3 and MAPK signaling pathways. The observation of Stat3 activation by IL-6 is consistent with other reports that IL-6 stimulates prostate cancer cell growth through activation of the Stat3 signaling pathway (16, 18, 22, 27), and IL-6-induced activation of Stat3 in LNCaP cells increases AR-mediated gene activation in an androgen-independent but IL-6-dependent manner (20). IL-6 can activate erbB2 receptors, leading to activation of the MAPK pathway (22, 28). We also demonstrated that overexpression of IL-6 in LNCaP cells has less effect on the activation of Akt phosphorylation, which is different to the report that IL-6 can lead to activation of PI3K-Akt resulting in prevention of programmed cell death in human prostate cancer cell lines (18, 25, 29). The differential effects of IL-6 on the various signaling pathways (Stat3, MAPK, and PI3K-Akt) in LNCaP cells resulting in cell proliferation, differentiation, and survival are intriguing and are currently under intensive investigation.

PSA is a marker for prostate cancer, and the rise of the levels of PSA in the serum is an important indicator of prostate cancer progression. Several reports have indicated that IL-6 enhances PSA expression in LNCaP cells *in vitro* (17, 20–22), possibly through activation of Stat3 signaling (20). This is consistent with our finding that overexpression of IL-6 in LNCaP cells enhances endogenous PSA expression. In addition, we further demonstrated that overexpression of IL-6 induces PSA secretion to the serum in the castrated male nude mice, indicating that PSA levels induced by IL-6 are accompanied by LNCaP tumor growth in castrated male nude mice, similar to the clinical observation that rising PSA levels are a potential indicator of hormone-refractory prostate cancer. We have also demonstrated that overexpression of IL-6 enhances AR-ARE DNA binding activity and enhances AR nuclear translocation in LNCaP cells, which is consistent with the report that IL-6 increases AR expression in LNCaP cells (17).

One of the limitations of this study may be that IL-6 affects only LNCaP cells. LNCaP is an androgen-sensitive human prostate cancer cell line expressing a functional but mutant AR, which has been widely used for the study of prostate cancer. We are currently investigating the effect of IL-6 on androgen responsiveness in androgen-sensitive human prostate cancer cells

expressing a wild-type AR. Nevertheless, this study provides the first experimental evidence that IL-6 induces the transition of prostate cancer from an androgen-dependent to an androgen-independent phenotype, which corresponds to the induction of PSA expression through activation of AR. The androgen-independent phenotype induced by IL-6 in LNCaP cells is accompanied by significant activation of the Stat3 and MAPK signal pathways.

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The Prostate 52:123–129 (2002)

Stat3 Enhances the Growth of LNCaP Human Prostate Cancer Cells in Intact and Castrated Male Nude Mice

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BACKGROUND. Prostate cancer frequently progresses from an initial androgen dependence to androgen independence, rendering the only effective androgen ablation therapy useless. The mechanism underlying the androgen-independent progression is unknown. Stat3, a member of the family of signal transducers and activators of transcription, is activated in numerous cancers, including prostate. This study is to investigate the role of Stat3 activation in the growth of prostate cancer cells.

METHODS. A constitutively active Stat3 was ectopically expressed in androgen-sensitive LNCaP prostate cancer cells and resulting stable clones expressing activated Stat3 were isolated. The effect of Stat3 activation on LNCaP cell growth in response to androgen in vitro and in vivo was examined.

RESULTS. We show that the levels of activated Stat3 are associated with the progression of androgen-independent prostate cancer. Activation of Stat3 in androgen-sensitive LNCaP prostate cancer cells results in enhancement of tumor growth in both intact and castrated male nude mice and enhances androgen receptor-mediated prostate specific antigen expression.

CONCLUSIONS. These findings demonstrate that intracellular signaling mediated by Stat3 can enhance the growth of androgen-sensitive human LNCaP prostate cancer cells in both intact and castrated male nude mice. *Prostate 52: 123–129, 2002.* © 2002 Wiley-Liss, Inc.

KEY WORDS: **Stat3; androgen-independence; androgen receptor; prostate cancer**

INTRODUCTION

Hormone-refractory prostate cancer refers to a resistance to androgen ablation therapy, the only effective systemic therapy available for advanced prostate cancer. Almost all patients with advanced prostate cancer respond initially to androgen ablation therapy. However, virtually every patient will relapse to hormone-refractory disease due to the growth of androgen-independent cancer cells. There is growing evidence supporting the concept that the paracrine and autocrine loops mediated by growth factors and cytokines play an important role in acquisition of hormone independence [1,2]. Stat3, a member of Janus Kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) signaling pathway, is implicated in many cytokine-, hormone-, and growth factor–mediated signaling pathways to regulate a variety of biological responses, including development, differentiation, cell proliferation, and survival [3,4].

Constitutively activated Stat3 protein is found in various types of tumors, including leukemia, breast,

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head and neck, and prostate [5–10]. In addition, constitutively activated Stat3 (a mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3) induces cellular transformation and tumor formation in nude mice [11]. These results suggest that Stat3 may function as an oncogene and play a critical role in transformation and tumor progression. Here, we investigated the effect of Stat3 activation on the growth of androgen-sensitive LNCaP cells in the intact and castrated male nude mice.

MATERIALS AND METHODS

Cell Culture and Plasmids

Human LNCaP prostate cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The LNCaP cells were passaged twice weekly, and the passage number approximately eight were used for all of the studies. Androgen-insensitive LN95, LN96, LN97, and LN98 human prostate cancer cells [12] were maintained in phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped serum (Hyclone, CA). HeLa cells were routinely maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% FBS. The cells were grown at 37°C in 5% CO_2 and 95% air. The plasmid (pSG5-wtAR) containing the wild-type full-length sequence of human androgen receptor (AR) was kindly provided by Dr. Chawnshang Chang, University of Rochester, NY. The plasmid containing the constitutively active form of Stat3c was provided by Dr. James Darnell, Jr., The Rockefeller University, NY [11]. Prostate specific antigen (PSA) regulatory element containing an 822-bp enhancer [13] and a 620-bp promoter [14] was generated by PCR amplification of Hirt DNA from LNCaP cells by using primers for the enhancer (5′ primer, 5′-GCGGTACCCTGCAGAGAAT; and 3′ primer, 5′-GGATCCCCATGGTTCTGTC), and for the promoter (5′ primer, GGATCCTTGGATTTTGAAAT; and 3′ primer, 5′-GGTCTAGAAAGCTTGGGGCT). The PCR products of the amplified enhancer and promoter were gel purified, kinased, and inserted into the *Eco*RV site of pBluescript KS + vector, separately. The PSA regulatory element was generated by inserting the enhancer (cut out with *Bam*HI from the pBluescript KS + construct) in front of the promoter at the *Bam*HI site of the pBluescript construct containing the promoter. The PSA reporter construct (pAAV-PSA-Luc) was generated by insertion of the 1.4-kb fragment of the PSA enhancer and promoter in place of the CMV promoter of the pAAV-CMV-Luc construct [15].

Luciferase Assay

Twenty-four hours before transfection, 3×10^5 cells were plated in a six-well plate in phenol red-free DMEM containing 5% dextran-coated charcoal-stripped FBS (CS-FBS). Cells were transfected with a total amount of 5 μg of DNA by using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. The total amount of plasmid DNA used was normalized to 5 μg/well by the addition of empty plasmid. Three hours later, the DNA:liposomes mixture was removed and cells were treated with phenol red-free medium containing 5% CS-FBS with either 10 nM dehydrotestosterone (DHT, Sigma, St. Louis, MO) or in the absence of DHT. Cell extracts were obtained 36 hr later, and luciferase activity was assayed by using the Luciferase Assay System (Promega, Madison, WI). Protein concentration in cell extracts was determined by Coomassie Plus protein assay (Pierce, Rockford, IL). Luciferase activities were normalized by protein concentrations of the samples. All transfection experiments were performed in triplicate wells and repeated at least four times.

Transfection and Northern Blot

Transfections with the plasmid expressing the constitutively active form of Stat3c or empty vector were performed by using Superfect (Qiagen, Valencia, CA) according to the manufacturer's protocol. Stable clones were selected in 800 μg/ml G418 and maintained in 300 μg/ml G418.

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, Rockville, MD). Twenty micrograms of each sample was electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 1.2-kb *Bam*HI fragment of the PSA cDNA was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) by using the Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out during 3 hr at 65°C in Rapid-hyb buffer (Amersham). Membranes were washed for 15 min at 65°C in 2 × SSC, 0.1% sodium dodecyl sulfate (SDS; twice), 0.5 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio-Rad, Hercules, CA).

Electromobility Shift Assay

Whole cell extracts were prepared and electromobility shift assays (EMSAs) were performed as described previously [8]. For supershift analyses, the cell extracts were preincubated with antibody specifically

against Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA). The protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel in 1 × TBE (90 mM Tris-borate, 2 mM EDTA) at room temperature, and the results were analyzed as above.

Western Blot

Whole cell extracts were obtained by lysing the cells in RIPA buffer (1 × phosphate buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, with freshly added protease inhibitors: 0.1 mM phenylmethyl sulfonyl fluoride, 1 mM sodium orthovanadate, 30 μl/ml aprotinin). Whole cell extracts were resolved in 12.5% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% milk in PBS-0.1% Tween 20, membranes were incubated for 1 hr at room temperature with anti-AR rabbit polyclonal antibody (Santa Cruz Biotechnology) or anti-FLAG antibody (Sigma), or anti-phospho-Stat3 antibody (Cell Signaling Technology, MA) diluted in 1% milk in PBS-Tween. After secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

In Vivo Assays

The mice were injected in the flank with 3×10^6 cells resuspended in Matrigel diluted 1:1 in complete culture medium. The volume of the growing tumors was estimated by measuring their three dimensions (length × width × depth) with a caliper.

PSA Protein Analysis

PSA secretion was quantitated by PSA immunoradiometric assay (Beckman Coulter, Fullerton, CA) of tissue culture supernatant. Equal numbers of cells were plated in phenol red-free RPMI containing 10% FBS. Cells were allowed to attach for 24 hr, then the medium was changed to phenol red-free medium supplemented with 10% charcoal-stripped serum. After another 2 days, 50 μl of supernatant was assayed for PSA.

Statistical Analysis

Values were expressed as the mean ± SE. Statistical analyses were performed by one-way analysis of variance, followed by the Student-Newman-Keuls test for multiple comparisons, with a $P < 0.05$ being considered significant.

RESULTS AND DISCUSSION

Stat3 Activation Is Associated With Androgen-Independent Progression

We first tested whether Stat3 activation is associated with the progression of androgen-independent prostate cancer. We analyzed Stat3 activity by EMSA and the expression of phosphorylated Stat3 protein in androgen-sensitive LNCaP human prostate cancer cells and androgen-insensitive sublines (LN95, LN96, LN97, and LN98) derived from LNCaP cells [12]. LNCaP sublines (LN95, LN96, LN97, and LN98) were derived from LNCaP cells after chronic androgen deprivation in vitro, which produces androgen-insensitive clones [12]. All of the LNCaP sublines retain the AR and produce PSA even in the absence of androgen [12]. The androgen-insensitive LNCaP sublines grew readily in both castrated and intact male nude mice compared with no tumors formed in wild-type LNCaP animals subcutaneously [12]. Both the levels of Stat3 activity and phosphorylated Stat3 protein were increased in the androgen-insensitive sublines compared with that of the parental LNCaP cell line (Fig. 1). These results suggest that androgen-insensitive growth is associated with increased levels of Stat3 activity in LNCaP human prostate cancer model.

Stat3 Activation Enhances LNCaP Cell Growth In Vitro and In Vivo

Because LNCaP cells have minimal levels of Stat3 activity, we tried to determine whether elevation of

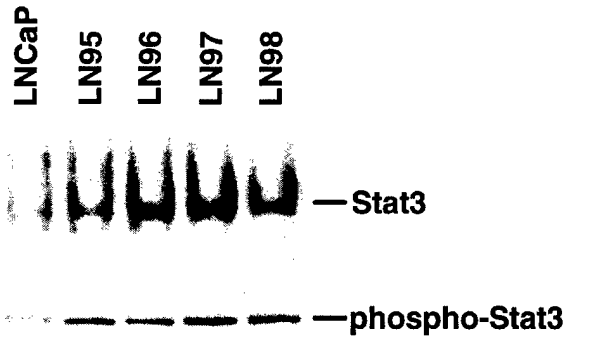

Fig. 1. Stat3 activation is associated with androgen-independence. **Top panel:** Stat3 DNA-binding activity in LNCaP and LN series cell lines. Whole cell extracts (20 μg) were subjected to EMSA by using a ^{32}P-labeled oligonucleotide probe containing the consensus binding motif for Stat3. **Bottom panel:** Stat3 tyrosine phosphorylation (phospho-Stat3) in LNCaP and LN series cell lines. Forty micrograms of whole cell protein extract was analyzed by Western blot by using antibodies specific against phosphotyrosine Stat3 (Tyr-705) antibody.

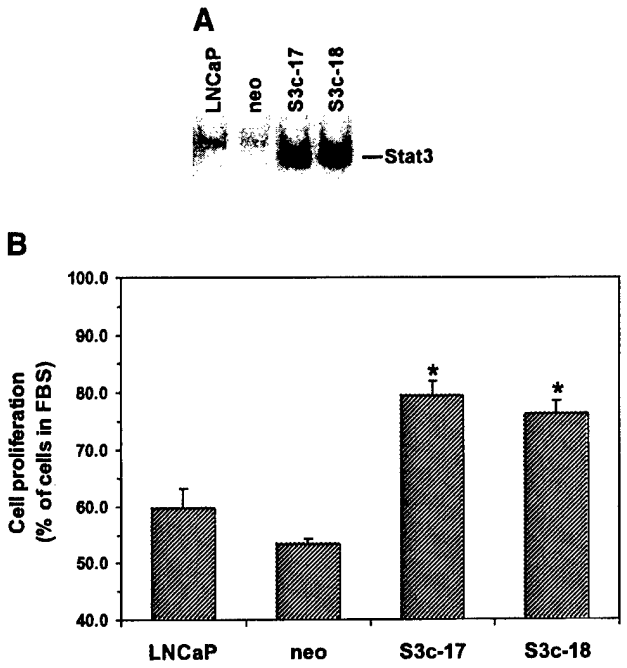

Fig. 2. Stat3 enhances androgen-independent growth in vitro. **A:** Stat3 DNA binding activity in Stat3-overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by EMSA. **B:** Effect of Stat3 on LNCaP cell growth in the presence and absence of androgen in vitro. Cells were cultured in RPMI-1640 supplemented with either 10% FBS or 10% charcoal-stripped FBS. Cell proliferation values in charcoal-stripped FBS were expressed as percentage relative to the complete FBS. $*P < 0.05$.

Stat3 activity will enhance LNCaP cell growth in vitro and in vivo. We introduced a constitutively activated Stat3 into the androgen-sensitive LNCaP cells. Two independent clones overexpressing activated Stat3 were selected (Fig. 2A). The growth of these cells in normal serum and in androgen-deprived serum was compared. The growth of LNCaP cells and neo vector alone control cells in culture was reduced by approximately 50% after 48 hr in androgen-deprived charcoal-stripped serum compared with that in the normal serum (Fig. 2B). In both clones of LNCaP cells overexpressing Stat3, however, there was only a 20% decrease in growth under these androgen-deprived conditions compared with growth in normal serum (Fig. 2B), suggesting that activated Stat3 can enhance the growth of LNCaP cells in the absence of androgen in vitro.

LNCaP is a human prostate cancer cell line derived from supraclavicular lymph node metastases [16,17]. These cells express mutant but functional androgen receptors and exhibit androgen-sensitive phenotype [16,17]. Igawa et al. reported that the aggressiveness and androgen responsiveness of LNCaP cells can be altered by culturing the cells continuously in vitro [18]. LNCaP cells in the early passages are usually less aggressive (i.e., lower tumorigenic in vivo) and higher androgen responsiveness in vitro and in vivo than that of the cells in their late passages [18]. The parental LNCaP cells used in the present study are in their early passages (see Materials and Methods section). The parental LNCaP cells and vector control Neo clone did not grow any detectable tumor in both intact (within 40-day observation period) and castrated (within 60-day observation period) male mice (Fig. 3B). We next tested the effect of Stat3 activation on LNCaP tumor growth in both intact and castrated male nude mice. Eight-week-old male nude mice were randomly divided into two groups, one left intact, another group received surgical castration. Three days after castration, intact or castrated male nude mice were injected subcutaneously with LNCaP cell clones overexpressing Stat3. For the two independent Stat3-overexpressing clones, tumors became apparent at the site of injection within 20 days in the intact male mice and within 30 days in the castrated male mice (Fig. 3A). There was a delay of the latency for tumor formation in the castrated male mice compared with the intact male mice (Fig. 3B). Western blot analysis of protein extracts derived from Stat3-overexpressing tumors in both intact and castrated male mice revealed high levels of FLAG-tagged Stat3 protein (Fig. 3C), indicating that the growing tumor cells continue to express FLAG-tagged Stat3 plasmids and are derived from human LNCaP cells. These results demonstrate that Stat3 activation not only enhances prostate cancer cell tumor growth in vivo, but also promotes tumor growth in the androgen-deprived castrated male nude mice.

Stat3 Enhances AR-Mediated Gene Expression Independent of Androgen

To determine whether Stat3 activation affects AR signaling, we tested the effects of Stat3 on the expression of endogenous PSA, a well-characterized prostate specific antigen whose transcription is strictly regulated by androgen [19]. The levels of PSA mRNA expression from LNCaP cell clones overexpressing activated Stat3 were increased compared with the parental LNCaP cells (Fig. 4A). To examine the effects of Stat3 activation on the expression of PSA upon androgen withdrawal, we measured PSA protein secretion in phenol red-free medium supplemented with the charcoal-treated serum. As shown in Figure 4B, the levels of PSA protein expression were increased in the Stat3-overexpressing LNCaP

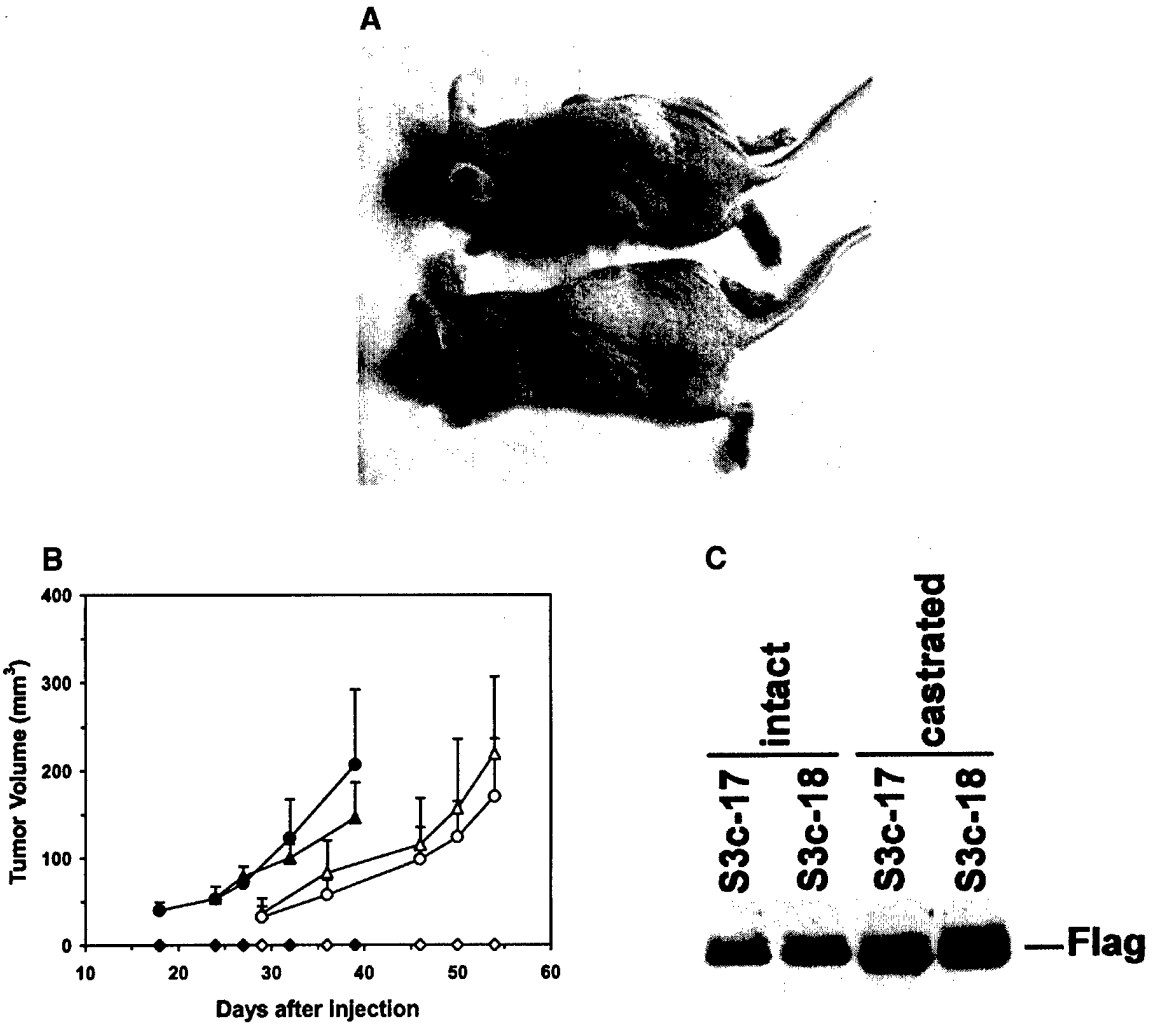

Fig. 3. Stat3 induces androgen-independent growth in vivo. **A:** Stat3-overexpressing clone (S3c-18) developed tumors vs. parental LNCaP cells, which did not grow any tumors in the castrated male nude mice. **B:** Tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone (◇) or clones that overexpress activated Stat3 (S3c-17, triangles; S3c-18, circles) were injected into the intact (filled symbols) or castrated (open symbols) male nude mice (n = 10 for each condition). **C:** Western blot analysis of Flag-tag expression in cell extracts from Stat3-derived tumors in the intact and castrated male nude mice.

subclones compared with the parental LNCaP cells and vector controls in androgen-deprived conditions, indicating that Stat3 can partially replace androgen function in activation of the AR-mediated PSA gene expression. Tumors expressing Stat3 also produced high levels of circulating PSA in the serum (average, 32 ng/ml per gram of tumor) in the castrated male mice.

To determine the effect of Stat3 activation on AR-mediated gene transcription, we transiently transfected LNCaP cells with a luciferase reporter linked to the androgen-responsive promoter of PSA and various amounts of expression vectors encoding the constitutively active Stat3 [11]. To compare the effect of Stat3 on PSA promoter activity in the presence and in the absence of the androgen, the cells were then cultured in phenol red-free medium supplemented with the charcoal-stripped serum either in the presence of 10 nM of dehydrotestosterone (DHT) or in the absence of DHT. After 24 hr, cells were harvested and luciferase activities were determined. As shown in Figure 5A, Stat3 activated the PSA-luc reporter in a concentration-dependent manner in the absence of androgen, suggesting that Stat3 activates PSA tran-

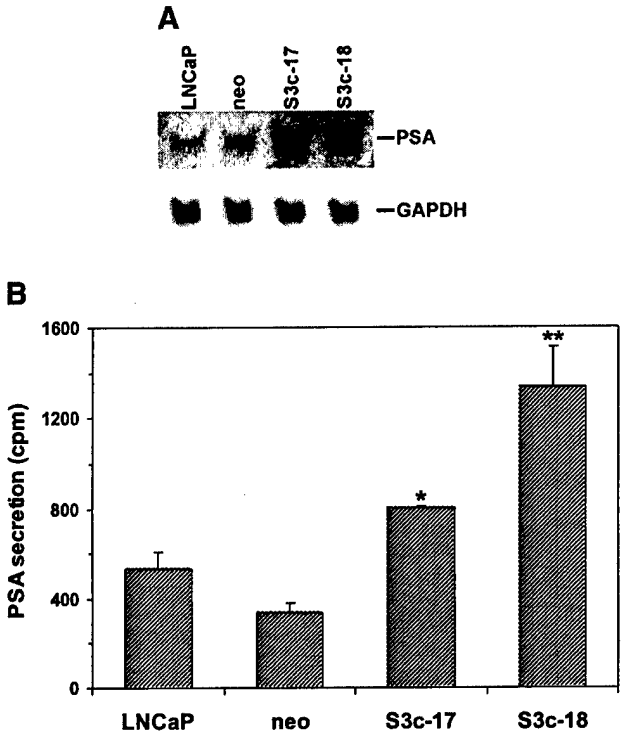

Fig. 4. Stat3 enhances prostate specific antigen (PSA) expression. **A:** PSA mRNA expression in Stat3-overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by Northern blot analysis by using 20 μg of total RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a control for equal loading. **B:** PSA protein secretion in the absence of androgen. PSA secretion was quantitated by PSA immunoradiometric assay of 50 μl of supernatant of cell culture in phenol red-free RPMI containing 10% charcoal-stripped serum. $*P < 0.05$; $**P < 0.01$.

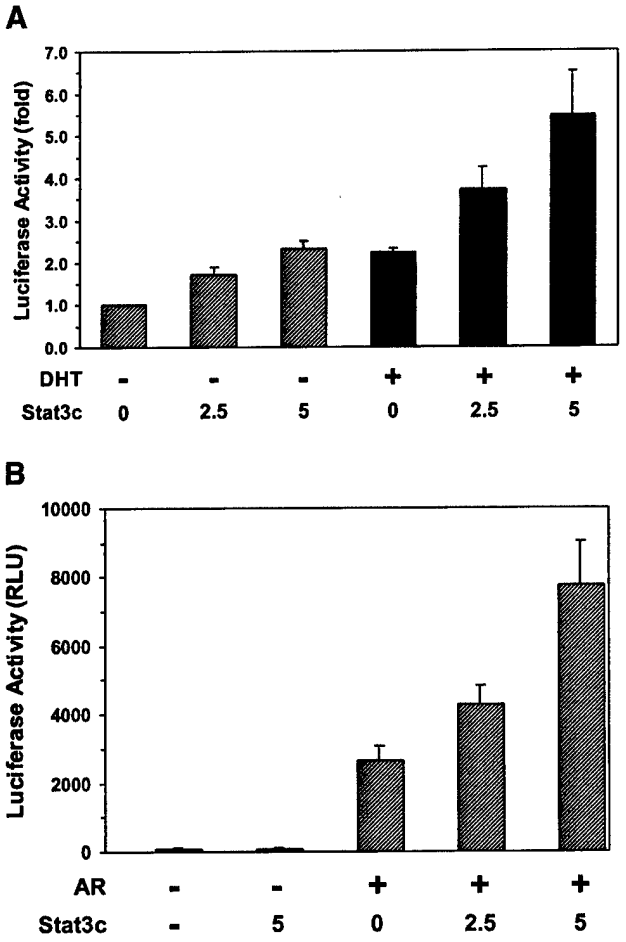

Fig. 5. **A:** Effect of Stat3 on prostate specific antigen (PSA) promoter activity in the absence of dehydrotestosterone (DHT) and in the presence of 10 nM of DHT. LNCaP cells were transiently transfected with PSA-luc reporter and increasing doses (0, 2.5, 5 μg) of Stat3 expression plasmid. Total DNA content was kept constant in all wells. **B:** The effect of Stat3 on PSA transcription requires androgen receptor (AR). HeLa cells transiently transfected with or without AR expression plasmid, PSA-luc reporter, and increasing doses (0, 2.5, 5 μg) of Stat3c expression plasmid in the presence of 10 nM of DHT. Total DNA content was kept constant in all wells. The luciferase activity was measured. Results are displayed as the average of four independent experiments. RLU, relative light units.

scription in a ligand-independent manner. Addition of 10 nM of DHT-enhanced PSA-luc reporter activity induced by Stat3 (Fig. 5A). To test whether the effects of Stat3 on PSA transcription require AR, we transiently transfected AR-negative HeLa cells [20], with or without an AR expression vector, plus a luciferase reporter with the androgen-responsive promoter of PSA (PSA-Luc), and increasing amounts of expression vector encoding the constitutively active Stat3c. Cotransfections lacking the AR failed to result in activation of PSA-luc reporter gene (Fig. 5B), suggesting that the effects of Stat3 on androgen responsive gene expression are dependent on AR.

Our findings demonstrate that increased Stat3 activity promotes tumor growth of androgen-sensitive LNCaP prostate cancer cells in both intact and castrated male nude mice and enhances AR-mediated PSA expression both in the presence and absence of androgen.

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The Prostate 51:241–246 (2002)

Stat3 Activation in Prostatic Carcinomas

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BACKGROUND. Activated Stat3 is found in various types of immortal cell lines and cancers. We and others have previously demonstrated that Stat3 is constitutively activated in rat and human prostate cancer cell lines, and that Stat3 activation is involved in IL-6-mediated signaling transduction in prostate cancer cells. The aim of this study is to examine quantitative Stat3 activity in benign and malignant human prostate tissues and analyze the association between Stat3 activity levels and the clinical and pathologic parameters.

METHODS. Stat3 activity levels were analyzed in a total of 104 human primary prostate tissues using electromobility shift assay and immunohistochemical staining for phosphorylated Stat3. The tissue samples used were 42 prostate carcinomas, 42 matched normal prostate tissues from patients with prostatic adenocarcinoma (normal adjacent to tumor), and 20 normal prostate tissues from organ donors.

RESULTS. Significantly higher levels of constitutive Stat3 activity were detected in both prostate carcinomas and the matched normal prostate tissues adjacent to tumors compared to the normal prostates from donors without prostate cancer. There was no significant difference of Stat3 activity in foci of tumor and normal prostate tissue adjacent to tumor. No correlation was seen between Stat3 activity and Gleason grade or serum PSA levels in samples from prostate carcinomas.

CONCLUSIONS. These results indicate that Stat3 is constitutively activated in prostate cancer. The high level of Stat3 activity in both the prostate carcinomas and the normal prostate tissues adjacent to tumors suggests that Stat3 activation may occur before detectable histological alterations of the prostate. *Prostate 51: 241–246, 2002.* © 2002 Wiley-Liss, Inc.

KEY WORDS: **Stat3; IL-6; prostate**

INTRODUCTION

The development of a clinical prostate cancer from a normal prostatic glandular cell requires multiple transformation events. The molecular mechanism associated with the initial development of histological prostate cancer is incompletely understood.

Stat3, a member of JAK-STAT signaling pathway, is a latent transcription factor which is activated by phosphorylation [1,2]. Activated Stat3 dissociates from the receptor, and forms homo- and hetero-dimers. These dimers translocate to the nucleus and bind to cognate DNA response elements, thus activating target gene transcription [1]. To date, STAT proteins comprise a family of seven known transcription factors, denoted Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6 [2]. Selective STATs can be activated by a number of different cytokines and growth factors.

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Constitutively activated Stat3 protein is found in various types of tumors including leukemia, breast, and head and neck [3–5]. Activation of Stat3 has been observed in cells transformed in vitro with v-src and abl oncogenes [6,7]. Furthermore, constitutively activated Stat3 (a mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3) induces cellular transformation and tumor formation in nude mice [8]. These results suggest that Stat3 may function as an oncogene and play a critical role in transformation and tumor progression.

We previously demonstrated that IL-6 stimulates prostate cancer cell growth accompanied by activation of Stat3 [9]. Recently, we demonstrated that cells derived from both rat and human prostate cancers have constitutively activated Stat3, and that Stat3 activation is associated with malignant potential [10]. In addition, blockade of activated Stat3 by ectopic expression of a dominant-negative Stat3 in human prostate cancer cells that expresses constitutively activated Stat3 significantly suppresses their growth in vitro and in vivo [10]. These results suggest that activation of Stat3 signaling pathway is critical for the growth of prostate cancer cells.

In this study, we examined quantitative Stat3 activity in benign and malignant human prostate tissues and analyzed the association between Stat3 activity levels and the clinical and pathologic parameters.

MATERIALS AND METHODS

Patients and Tissue Samples

Tissues were obtained from patients undergoing prostatectomy at the University of Pittsburgh Medical Center. The prostatectomy specimens were either radical prostectomies, performed for prostate cancer, or donor cysto-prostatectomies, performed to obtain prostate tissues from tissue donors. These donors serve as true normal controls for analysis. Frozen tissues were used for this study.

A spectrum of Gleason scores was seen in the prostatic adenocarcinoma evaluated in these patients. One patient had a well-differentiated prostatic adenocarcinoma (Gleason score of 4), seventeen patients had moderately differentiate prostatic adenocarcinoma (Gleason scores 5 and 6), and 24 patients had a poorly differentiated prostatic adenocarcinoma (Gleason scores of 7, 8, and 9).

The pathologic TNM staging of the radical prostatectomies from these patients demonstrated 19 patients with a pathologic T2 stage and 23 patients with a pathologic T3 stage.

The estimation of the tumor size is based on both gross as well as microscopic evaluation. Many cases show well-defined tumor nodules that can be measured. However, light microscopic evaluation, and measurement of the tumor dimensions on the glass slide, is used to confirm the gross impression. Some cases show no obvious tumor on gross inspection. The microscopic measurement is used in these cases to document the size of the largest tumor nodule.

Electromobility Shift Assay (EMSA) and Supershift Assay

Whole cell extracts were prepared by homogenizing the tissues in high salt buffer (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM $Na_3P_2O_7$, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 420 mM NaCl, 20% glycerol, 1 μg/ml of leupeptin, and 1 μg/ml of aprotinin), followed by snap-freezing in ethanol/dry ice for 5 min and thawing on ice for 10 min. The freeze and thaw procedures were repeated again for a total of two times. The supernatant was then centrifuged and harvested. Protein concentrations were determined by Coomassie plus protein assay kit (Pierce, IL) according to the manufacturer's protocol. Whole cell extracts (20 μg) were incubated in a final volume of 20 μl [10 mM HEPES, pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μg/ml poly(dI-dC)] by EMSA with radiolabeled double-stranded Stat3 consensus binding motif 5′-GATCCT T C T G G G A A T T CCTAGATC (Santa Cruz Biotechnologies, CA) for 20 min at room temperature. For supershift analyses, the cell extracts were pre-incubated with antibody specifically against Stat3 (Santa Cruz Biotechnologies, CA). The protein-DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel containing 2.5% glycerol in 0.25 × TBE at room temperature and the results were autoradiographed.

TSU human prostate cancer cells were used as a positive control [10]. Quantitation of the amount of Stat3 DNA-binding activity in the "protein-DNA" bandshift was measured using PhosphorImager software (Molecular Dynamic, CA). A positive control lane with TSU cell protein incubated with the Stat3 probe was routinely run on each gel [10]. The PhosphorImager values on each sample were normalized to the level in the TSU cells run on each gel.

Immunohistochemical Staining

Immunohistochemical evaluation of Stat3 expression was performed using an antibody directed against phosphorylated Stat3 (the active form of Stat3). Formalin fixed, paraffin embedded tissues, cut at 4-micron thickness, were used.

The tissue sections were deparaffinized with xylene rinses and hydrated through graduated ethanols. The

slides were then placed in a 3% methanol/hydrogen peroxide bath to quench endogenous peroxidase for 15 min. An antigen retrieval step utilizing Biogenex Citra Antigen Retrieval solution according to the Biogenex pressure cooker protocol was incorporated. The slides were then placed in a Protein Blocking Agent commercially prepared by Immunon Shandon for 20 min.

The primary antibody had been purchased from New England Biolabs (NEB) and is a polyclonal, rabbit anti Phospho-Stat 3 (Tyr 705). The optimal dilution was run at 1:100 prepared in Biogenex Common Antibody diluted with an overnight incubation temperature of 4°C. Slides were washed in PBS with 0.2% Tween for 10 min.

The rabbit polyclonal IgG antibody was used as a negative control. The secondary antibody, Vector Biotinylated Anti-rabbit at a dilution of 1:200 was applied to the tissues for 30 min. The slides were washed again for 10 min in the PBS solution.

Avidin-Biotin Complex (ABC) purchased also from Vector as the Elite ABC Kit, made according to manufacturer's direction was applied to the slides for 30 min. Slides are washed for 10 min in PBS. The slides were then stained for 10 min utilizing Vector peroxidase diaminobenzidine substrate (DAB) kit, followed by 2 distilled water rinses, stained with Shandon Hematoxylin for 3 min, dehydrated though graduated ethanols, xylene baths, and mounted with Permount.

Positive controls used were cell pellets, embedded in paraffin, of TSU, a human prostate cancer cell line. This constitutively expresses a high level of Stat3. By experimentation, we realized that prostate cancer slides, from routine prostatectomy specimens, work equally well. Deeper levels from the same block were used as positive control, along with the TSU pellets. The intensity of staining was subjectively graded on a scale of 0–3, with 0 being absence of expression and 3 being very strong expression. A cell line (TSU), known to have a high level of expression of phosphorylated Stat3, was used as a positive control. This positive control staining intensity was considered a 3+.

Statistical Analysis

The significance of the differences between Stat3 activity and clinico-pathologic variables was evaluated using the Fisher exact test. The correlation of Stat3 activity with serum PSA levels was evaluated with regression analysis. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Eighty-four specimens from 42 matched primary prostate tumors were evaluated. The samples analyzed consisted of one sample each from an area of prostatic adenocarcinomas and "normal adjacent to tumors" from patients with different Gleason grades and TNM stage tumors. In addition, samples from 20 normal prostates (organ donors) without prostate pathology were assessed for Stat3 activation by EMSA. Figure 1A shows a representative EMSA analysis of Stat3 activity. We performed supershift analysis, using appropriate antisera, to verify that the Stat3 complex contained Stat3 (Fig. 1B).

Comparison of Stat3 activity between matched normal adjacent to tumor and tumor tissues revealed higher levels of activity in 43% tumor specimens (18 of 42), lower level of activity in 33% tumor specimens (14 of 42), and similar level of activity in 24% specimens (10 of 42). As shown in Figure 2, there is no significant difference of Stat3 activity between the matched normal adjacent to tumor and tumor samples. However, Stat3 activity was significantly increased ($P < 0.01$) in both matched normal and tumor specimens compared to that of the specimens from organ donors, who had no prostate pathology.

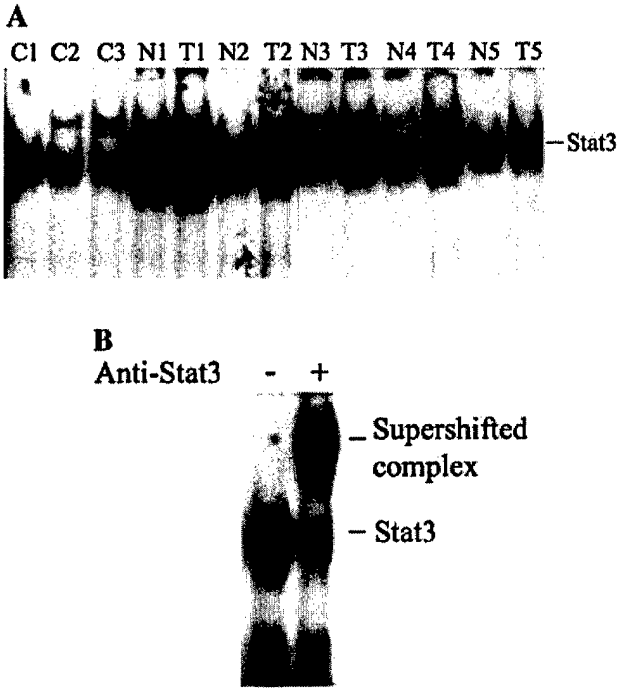

Fig. 1. **A**: Stat3 DNA-binding activity in human prostate tissues. Whole cell extracts (20 μg) were subjected to EMSA using a ^{32}P-labeled oligonucleotides probe containing the consensus binding motif for Stat3. C: normal prostate tissues from three individual organ donors; N: matched normal adjacent to tumor; T: prostate carcinomas. **B**: Supershift assay. The whole cell extracts from prostate tumor tissue were preincubated with anti-Stat3 antibody. The position of the Stat3 and the supershifted complexes are indicated on the right.

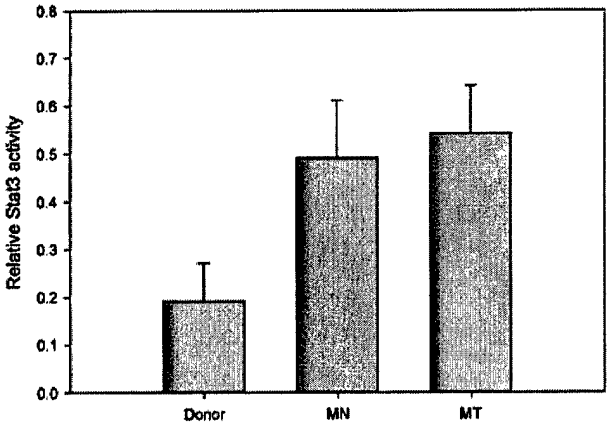

Fig. 2. Cumulative results of Stat3 DNA-binding activity in normal prostate tissues from 20 individual organ donors (donor), 42 matched normal tissue adjacent to tumor (MN), and 42 matched prostate carcinomas (MT).

Of 42 evaluated prostate cancer tissues, the average level of Stat3 activity increased with increasing Gleason score, but this difference did not achieve statistical significance (Table I). The relationships between Stat3 activity, pretreatment PSA levels, Gleason score, and tumor size are presented in Table II. The average levels of pretreatment PSA from Stat3 activity T > N group are higher than that of T < N and T = N groups. But, this difference did not achieve statistical significance.

Increased Stat3 activation can occur through several potential mechanisms, including elevated constitutive levels of Stat3 protein and increased Stat3 phosphorylation. Stat3 activation is accompanied by phosphorylation at Tyr705, which induces dimerization, nuclear translocation, and DNA binding. To localize the activated Stat3 in prostate cancer tissue sections and to determine whether the increase in constitutive Stat3 activation is associated with increased protein expression, immunohistochemical staining, using phospho-Stat3 antibody (Phospho-Stat3 [tyr-705], New England Biolabs, Beverly, MA), was performed on formalin fixed paraffin embedded tissues. The cases selected for the study were the same as those on which gel shift data was available.

TABLE I. Stat3 Activity and Gleason Score

	GSS < 6	GSS = 7	GSS > 8
Sample no.	16	17	9
Stat3 activity (total)	0.48	0.50	0.52
Stat3 activity in MN	0.47	0.48	0.50
Stat3 activity in MT	0.48	0.50	0.53

TABLE II. Stat3 Activity, Gleason Grade, and PSA

Stat3 activity	No.	%	Pretreatment PSA (ng/ml)	Gleason score	Tumor size
MT < MN	14	33	7.6 ± 4.0	6.6	1.5
MT = MN	10	24	5.4 ± 3.2	6.4	1.7
MT > MN	18	43	8.7 ± 3.8	6.5	1.7

Immunohistochemical staining showed either weak or negative expression of phosphorylated Stat3 in the donor tissues (0 to 1+ level of expression). Moderate to strong (2+ and 3+) expression of Stat3 was seen in the foci of prostatic adenocarcinoma and in the areas of "normal" prostate adjacent to tumor tissue. (Fig. 3). The phosphorylated Stat3 was expressed in the nucleus of the glandular epithelial cells of both the adenocarcinomas and the normal prostate adjacent to tumor (Fig. 3). In general, immuno-histochemical staining and the gel shift assay showed very similar pattern of Stat3 expression.

The potential mechanism responsible for the Stat3 activation in prostate cancer remains unclear. Stat3 is a major transcription factor mediated Interleukin-6 (IL-6) signaling transduction [11]. Expression of IL-6 and its receptor has been consistently demonstrated not only in human prostate cancer cell lines but also in human prostate carcinoma and benign prostate hyperplasia obtained directly from patients [12–14]. IL-6 levels are elevated in the sera of patients with metastatic prostate cancer and hormone-refractory prostate cancer compared to levels in sera from controls [15]. It has been demonstrated that IL-6 stimulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor through Stat3 dependent signal pathway in LNCaP cells [9,16,17].

IL-6 belongs to the IL-6 family of cytokine, which includes leukemia inhibitory factor (LIF/IL-6), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), IL-11, and cardiotrophiu-1 (CT-1) [11]. Signal transducer Gp130, a common subunit of the receptor complexes for the IL-6 family of cytokines, transmits the cytokine-mediated signalings from the membrane to the nucleus through the Stat3 signaling transduction pathway [11]. Recent studies have demonstrated that IL-11 and IL-11 receptor are expressed in normal and malignant prostate cell lines [18]. In addition, the levels of IL-11 receptor and Gp130 expression are significantly elevated in prostate carcinomas, which correlated with the increased levels of Stat3 activity [18].

In conclusion, we demonstrated that Stat3 activity is significantly increased in both prostate cancer and their adjacent normal prostate tissues compared to the

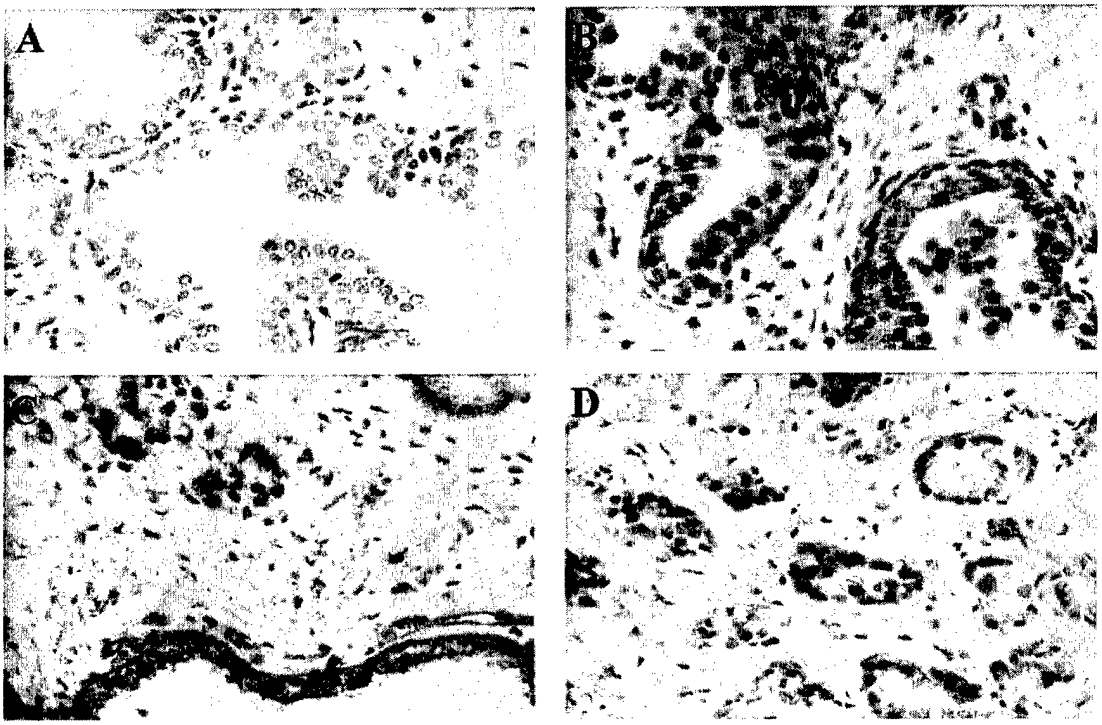

Fig. 3. Stat3 activation in prostate cancers. Tissue sections were immunohistochemically stained for phospho-Stat3 antibody (Phospho-Stat3 [tyr-705], New England Biolabs) using peroxidase diaminobenzidine substrate (DAB) kit. Sections were counterstained with Shandon Hematoxalin. **A**: normal prostate from donor; (**B**) matched normal tissue adjacent to tumor; (**C**) matched normal tissue and adjacent tumor; (**D**) prostate carcinomas.

normal prostates from patients without cancer using both EMSA and immunohistochemical methods. There does not appear to be a statistically significant difference in Stat3 expression between normal adjacent to tumor and tumor tissues. The high level expression of Stat3 in both prostate carcinomas and normal tissues adjacent to tumor suggest that activation of Stat3 may be an early event in prostatic epithelial carcinogenesis.

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Stat3 enhances transactivation of steroid hormone receptors

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Abstract

Background: Steroid hormone receptors (SHRs) are members of the superfamily of ligand-activated transcription factors that regulate many biological processes. Co-regulators act as bridging molecules between the SHR and general transcription factors to enhance transactivation of target genes. Previous studies demonstrated that Stat3 is constitutively activated in prostate cancer and can enhance prostate specific antigen (PSA) expression and promote androgen independent growth. In this study, we investigate whether Stat3 can enhance steroid hormone receptors activation.

Methods: CV-1 cells in which plasmids expressing androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR) or estrogen receptor (ER) were cotransfected with a constitutively active STAT3 mutant.

Results: Stat3 stimulates the transcriptional activity of all four SHR tested, AR, GR, PR and ER, in a hormone-dependent manner. Stat3 acts in a synergistic fashion with other coactivators such as SRC-1, pCAF, CBP, and TIF-2 on the transcriptional activity of these SHR. In addition, Stat3 significantly enhanced the sensitivity of androgen receptor in response to androgen. STAT3 did not affect the specificity of AR for other steroid hormones other than androgen or binding of AR to other hormone responsive elements.

Conclusions: These findings suggest that Stat3 can enhance the transactivation of AR, GR, PR and ER, and activated Stat3 could have a role in the development or progression of a hypersensitive AR.

Introduction

Steroid hormone receptors (SHRs) are members of a family of ligand-activated transcription factors that regulate many biological processes, including metabolism, reproduction, and development. In the absence of ligand such as androgen, glucocorticoid, progestin, and mineralocorticoid, the SHR maintains a cytosolic inactive state by association with heat shock proteins and/or other proteins such as corepressors. Upon ligand binding, the SHR undergoes conformational changes that involve releas e from the repressor proteins, and translocation to the nucleus where it can bind to specific hormone responsive sequences in the DNA of genes regulated by steroid hormones [1,2]. The mechanism by which SHRs affect the rate of RNA polymerase II-directed transcription likely involves the interaction of receptors with components of the transcription preinitialization complex. This interaction may be direct, or it may occur indirectly through the

action of coactivators, which act as bridging factors. To date, numerous coactivator molecules have been isolated and characterized, encompassing several different families [3,4]. Most of these cofactors are expressed in a wide variety of cell types and can interact with more than one type of nuclear receptor. The recent findings that members of the several different families of coactivators possess intrinsic histone acetyltransferase activity suggests that activated SHRs, and nuclear receptors in general, may also recruit these cofactors to remodel chromatin structure for better accessibility of the transcriptional machinery to DNA [5,6].

The JAK/STAT signaling pathway is involved in many cytokines, hormones, and growth factors mediated signaling pathways to regulate a variety of biological responses, including development, cell differentiation, proliferation and survival [7,8]. Once STAT proteins are activated by tyrosine-phosphorylation, form homo or heterodimers that are translocated to the nucleus, where they can bind to specific sequences in the DNA, thereby stimulating gene transcription. Similarly to nuclear receptors and to other transcription factors, STAT proteins can interact with coactivators to modulate their transcriptional activity [9–12]. Other reports have shown direct interactions between several members of the JAK/STAT signaling pathway with SHRs [13–16]. Stat3 is one of the seven members of the STAT family of proteins that has been shown to modulate the expression of several genes related to control cell cycle, proliferation and apoptosis, such as Cyclin D1, c-myc, and Bcl-xL, respectively [17]. Accordingly, alterations in the activity of STAT3 have been associated with cell transformation and cancer progression [18–20].

Prostate cancer is the most commonly diagnosed cancer, and the second leading cause of death from cancer in North American men. Prostate cells are dependant on androgen to keep their normal functions. Activation of androgen receptor (AR) in prostate cells is a key step in developing and progression of prostate cancer. Most patients respond initially to androgen deprivation or antiandrogen regiments, but eventually the tumor relapses in an androgen-independent stage with a poor outcome. Several possible mechanisms have been suggested to explain this activation of AR, including mutations in the gene encoding AR that alter the specificity for androgens, overexpression of the AR protein itself, crosstalk to other signal transduction pathways, and altered levels or activity of coactivators [21–23]. Several groups including ours have demonstrated a role of activated Stat3 in the proliferation and survival of prostate cancer cells by a mechanism that involves the AR [24–28].

In this study, we examined how Stat3 activates the SHRs including AR. We studied whether Stat3, a transcription factor itself, could act as a coactivator for AR and for other SHRs. We report here that a constitutively active form of Stat3 stimulates transcriptional activity of SHRs in a hormone-dependent manner. We also report that active Stat3 can act synergistically with other coactivators to stimulate SHRs transcriptional activity. Moreover, while Stat3 did not affect specificity of AR to other steroid hormones rather than androgen, or binding of AR to other HREs, Stat3 significantly increased the sensitivity of AR to androgen. These results can add more light to the mechanism by which SHRs, and AR in particular, regulate gene expression.

Results

Effect of Stat3 on AR-mediated transcriptional activity

Stat3 has been shown to enhance AR-mediated PSA expression and AR transcriptional activity in prostate cancer cells [16,25,28]. We used two well-characterized androgen-responsive luciferase reporter plasmids to study the effect of Stat3 on AR-mediated transcriptional activity. Both reporter plasmids contain several AREs that are required for androgen induction. As comparison, we used a saturating dose, 10 nM, of a synthetic androgen, R1881. CV-1 cells do not express endogenous AR, therefore, all experiments were performed in the presence of ectopic wild-type human AR. CV-1 cells showed a strong response to R1881 compared to vehicle-treated cells with both androgen-responsive reporters used (Fig. 1). Cotransfection of a plasmid expressing a constitutively active mutant Stat3, Stat3C, that forms homodimers and translocates to the nucleus without tyrosine phosphorylation, affected AR transcriptional activity in a dose-response manner (Fig. 1A) but only in the presence of hormone (Fig. 1B and 1C). Constitutively activated Stat3 also activates another androgen-responsive reporter contained the fragment -286/+28 of the rat Probasin (PB) promoter (Fig. 1C). Wild-type Stat3, that is in a latent cytosolic state without tyrosine phosphorylation nor dimerization, showed no additional stimulatory effect over the androgen-treated cells (Fig. 1)

Effect of STAT3 on transcriptional activity of other SHRs

Having demonstrated that Stat3 enhance AR-mediated gene transcription, we next investigated the role of Stat3 on transcriptional activation with other SHRs. For GR and PR, we used the same reporter plasmid as for AR, ARE-TATA-luciferase, since it shows promiscuous response to all three SHRs [30]. For ER, we used ERE-TATA-luciferase reporter that is specific for estrogen. CV-1 cells were also cotransfected with plasmids expressing ectopically GR, PR or ER. Like the AR, Stat3 enhances all three other SHRs tested in the presence of the corresponding hormone (Dex for GR, R5020 for PR, or E_2 for ER) (Fig. 2). Taken together, Stat3 enhances four different SHRs transcriptional activity in the presence of their

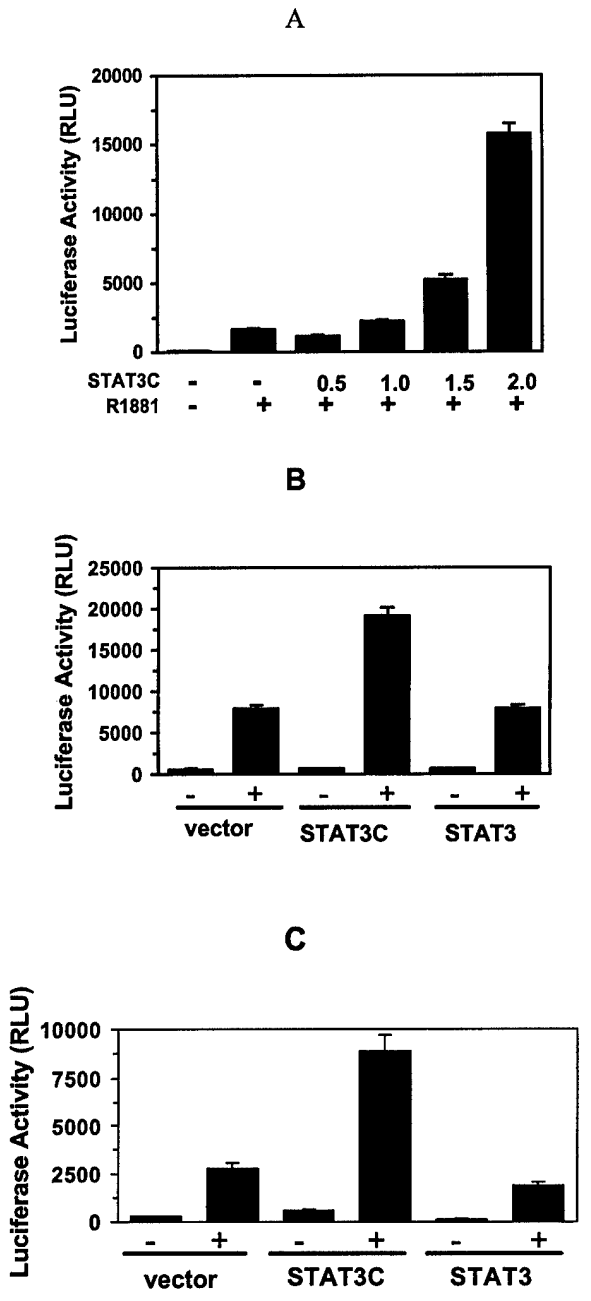

Figure 1

Active Stat3 stimulates androgen-induced AR transcriptional activity. CV-1 cells were cotransfected with AR responsive reporter plasmids, ARE-TATA-luciferase (**A, B**) or PB(-286/+28)-luciferase (**C**), AR, and Stat3C, S tat3, or empty vector. After transfection, cells were treated for 36–40 h with 10 nM R1881 or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

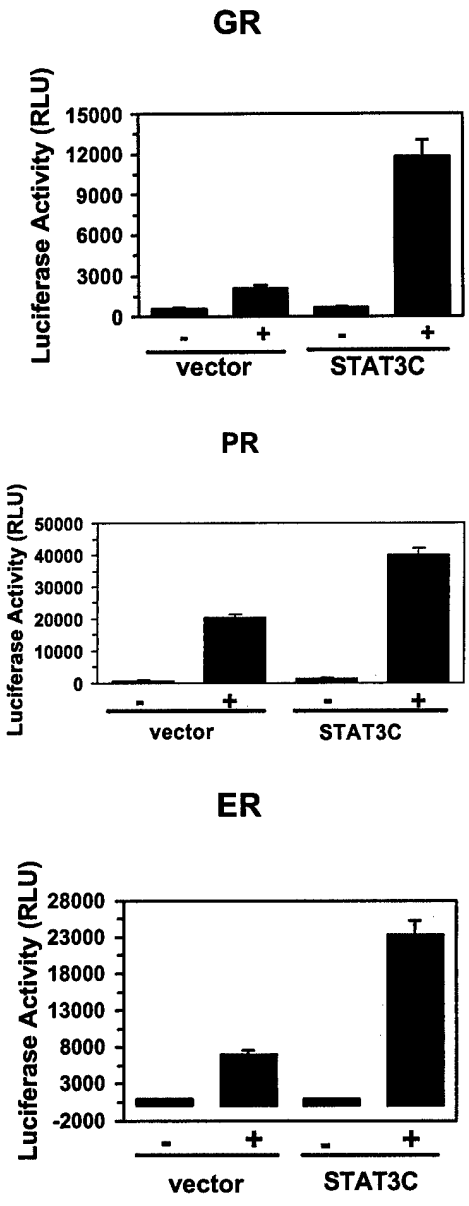

Figure 2

Active Stat3 stimulates hormone-induced transcriptional activity of other SHRs. CV-1 cells were cotransfected with specific luciferase reporter plasmids (ARE-TATA-luciferase for GR and PR, and ERE-TATA-luciferase for ER), their corresponding SHR expressing plasmid (GR, top; PR, middle; and ER, bottom), and Stat3C plasmid or empty vector. After transfection, cells were treated for 36–40 h with 10 nM of the corresponding hormone (Dex for GR; R5020 for PR; and E_2 for ER) or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

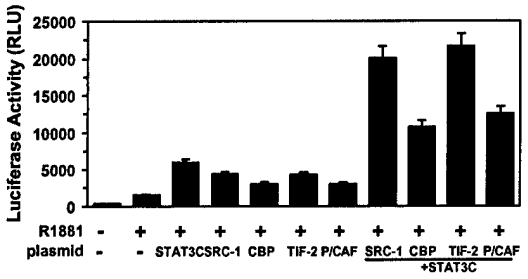

Figure 3
Active Stat3 stimulates AR transcriptional activity similarly as and synergistically with other coactivators. CV-1 cells were cotransfected with ARE-TATA-luciferase, AR, and Stat3C or different coactivators (SRC-1, CBP/p300, TIF2 or P/CAF), individually or in combination. All wells contained the same total amount of DNA. After transfection, cells were treated for 36–40 h with 10 nM R1881 or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

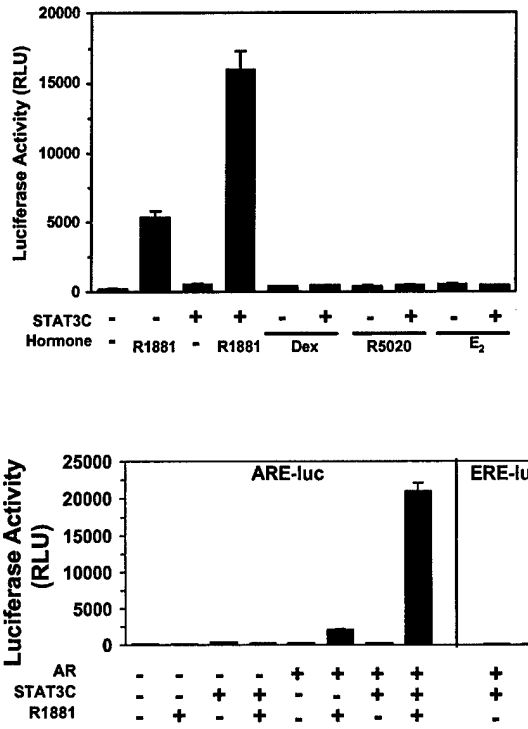

Figure 4
Stat3 does not affect specificity of AR for non-androgen steroid hormones or non-androgen responsive elements. (Top) CV-1 cells were cotransfected with ARE-TATA-luciferase, AR, and Stat3C or empty vector, and treated for 36–40 h with 10 nM each different steroid hormones (R1881, Dex, R5020, or E_2) or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. (Bottom) CV-1 cells were cotransfected with ARE-TATA-luciferase (left) or ERE-TATA-luciferase (right), AR, and Stat3C or empty vector, and treated for 36–40 h with 10 nM R1881 or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

corresponding ligand, suggesting that active Stat3 acts as a coactivator for nuclear receptors.

We next compared the coactivator activity of Stat3 to that of other known coactivators for AR. CV-1 cells were transfected with the same amount (2 μg) of plasmid expressing Stat3C, SRC-1, CBP/p300, TIF-2 or P/CAF, respectively. Cells transfected with either coactivator or Stat3C showed comparable and higher luciferase activity in response to R1881 than cells treated with hormone alone in the presence of empty vector (Fig. 3). None of the coactivators tested showed any luciferase activity in the absence of hormone (data not shown). Stat3 is comparable to other coactivators on AR transcriptional activity: SRC-1, CBP/p300, TIF2, P/CAF.

It has been reported that coactivators in combination can enhance transcriptional activity of SHRs compared to when they are delivered to cells individually [29,30]. Recently, Giraud et al [12] reported a direct interaction of Stat3 with SRC-1 and CBP/p300. We determined whether Stat3 could act in a synergistic/additive way when administered to cells simultaneously to other coactivators. As Fig. 3 shows, coexpression of active Stat3C simultaneously to SRC-1, CBP/p300, TIF-2 or P/CAF, resulted in a more efficient enhancement of the reporter transcription activity driven by AR as compared with any of the coactivators expressed separately. A similar enhancement in luciferase activity with simultaneous delivery of coactivators was observed with PR and ER (data not shown).

Stat3 did not affect the specificity of AR for non-androgen steroid hormones or non-AREs

Altered responses of AR include activation by non-androgen ligands or recognition of other hormone responsive elements different from AREs [22,23]. We studied if active Stat3 could be responsible for this behavior of AR. CV-1

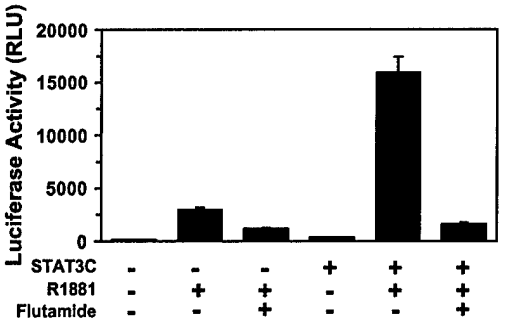

Figure 5
Inhibition of AR transcriptional activity by flutamide is not blocked by active Stat3. CV-1 cells were cotransfected with ARE-TATA-luciferase, AR, and Stat3C or empty vector, and treated for 36–40 h with 10 nM R1881 or 0.1% ethanol as vehicle in the presence or absence of 10 μM flutamide, in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are representative of three independent experiments. Data are expressed as mean ± S.E. of three independent experiments.

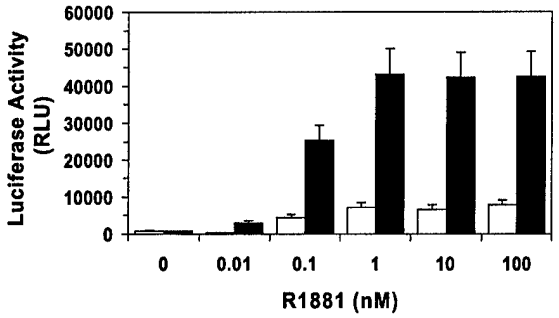

Figure 6
Active Stat3 modifies the sensitivity of AR for androgen. CV-1 cells were cotransfected with ARE-TATA-luciferase, AR, and Stat3C (solid bars) or empty vector (empty bars), and treated for 36–40 h with different doses of R1881 or 0.1% ethanol as vehicle, in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

cells were cotransfected with a reporter plasmid responsive to androgen (ARE-TATA-luciferase) in the presence or absence of AR and constitutively active Stat3C (Fig. 4 top) and cells were treated with different steroid hormones. Stat3C did not alter the specificity of AR for other steroid hormones, since it showed transcriptional activity only in response to androgen but not to Dex, R5020 or E_2 (Fig 4, top).

To test whether Stat3 could alter the specificity of AR to other HREs, CV-1 cells were cotransfected with reporter plasmids responsive to androgen (ARE-TATA-luciferase) or to estrogen (ERE-TATA-luciferase), in the presence or absence of AR and constitutively active Stat3C (Fig. 4 bottom). With ARE-TATA-luciferase reporter, luciferase activity was dependent on the presence of AR and androgen, without any effect of Stat3C on the basal levels of transcription (Fig 4, bottom left). Stat3C did not modify the sensitivity of AR for other hormone responsive elements, since there was no response on the AR transcriptional activity when we used an estrogen-specific responsive reporter plasmid, ERE-TATA-luciferase (Fig 4, bottom right).

Stat3 does not prevent inhibition of AR by the antiandrogen flutamide

Another altered response of AR is manifested in the so-called "flutamide withdrawal syndrome" or more recently broadened to "antiandrogen withdrawal syndrome". In this condition, it is shown a decrease in serum levels of PSA after removal of flutamide or other antiandrogens from the treatment regiment [33]. We studied if active Stat3 could be involved in this paradoxical effect of antiandrogens. CV-1 cells cotransfected with an androgen responsive reporter plasmid (ARE-TATA-luciferase), AR, in the presence or absence of constitutively active Stat3C, were treated with androgen and flutamide (Fig. 5). As expected, flutamide blocked substantially the induction of ARE-TATA-luciferase activity by R1881 in the absence of active Stat3C. Similarly, the inhibitory effect of the antiandrogen on AR was not prevented by the presence of constitutively active Stat3C.

Stat3 affects sensitivity of AR for androgen

Other mechanism by which AR could show an altered response results from an increase in its sensitivity to very low levels of androgen [22,23]. We tested the role of active Stat3 in this possibility cotransfecting CV-1 cells with an androgen responsive reporter (ARE-TATA-luciferase), AR, in the presence or in the absence of constitutively active Stat3C. Cells were then treated with different doses of androgen. Maximal transcriptional activity of AR was at 1 nM R1881 both in the presence and absence of Stat3C (Fig. 6). Stat3C enhanced luci ferase activity driven by AR even at the minimal dose of R1881 tested, 10 pM, which showed no luciferase activity in the absence of Stat3C.

Discussion

Regulation of gene transcription mediated by hormone-activated SHRs involves interaction with components of

the transcription complex. Coactivators are members of the transcription complex, which act as bridging factors to achieve optimal transcription activity. Coactivators are probably present in the nucleus in preassembled multicomplex units ready to associate to activated SHRs. The role of the coactivators could be just as bridging factors, although some of the members of the p160 family and CBP/p300 present histone acetyltransferase activity [34,35]. Stat3 is a member of the JAK/STAT signaling pathway, which is a transcription factor itself that after activation binds to specific sequences in DNA to regulate expression of genes related to proliferation, differentiation and cell survival. Constitutive activation of Stat3 has been associated with cell transformation and cancer progression. Previous data including from our group showed activation of AR in prostate cancer mediated by activation of Stat3 [24–28].

Here we show that constitutively active Stat3 enhances transcriptional activity of SHRs, AR, GR, PR, and ER, in a comparable extent to the stimulation elicit by other coactivators, and even more, in a synergistic manner to these other coactivators. This stimulation is independent of the DNA binding activity of Stat3, since none of the luciferase reporters responsive to SHRs used throughout this report contain the DNA sequence responsive to Stat3 [7]. We cannot rule out, however, that Stat3 is affecting an independent event that in turn activates SHR transcriptional activity. Nevertheless, direct protein-protein interaction has been documented by coimmunoprecipitation experiments between Stat3 and AR [16,25], GR [13,14], PR [36], and ER [37], suggesting that Stat3 might be involved directly in transcriptional activity elicit by SHRs. Moreover, Stat3 has also been directly associated with several coactivators, such as SRC-1 and CBP/p300 [9,12]. Recruitment of coactivators to nuclear receptors takes place via a common motif in the coactivators containing a core consensus sequence LXXLL (L, leucine; X, any aminoacid) [38]. Most of the coactivators contain more than one of these motifs, raising the question regarding specificity of these motifs to specific activating domains in the nuclear receptors. Stat3 also contains in its N-terminus one of this motifs (^{221}LAGLL^{225}) [39]. Moreover, Stat3 also presents a Ser at -2 position of the LXXLL sequence, which in the case of the coactivator TRBP defines selectivity for nuclear receptors [40]. Phosphorylation of Stat3 has been reported to occur only in ^{705}Tyr and in ^{727}Ser, allowing dimerization and full transactivating activity [41]. Whether this ^{219}Ser next to the LXXLL motif is involved in the coactivator activity of Stat3, and the interaction of Stat3 with other coactivators only takes place in the context of Stat3 transcription factor activity or also can be part of the general mechanism of the transcription complex formation requires further studies.

Activation of AR is a driving force in development and progression of prostate cancer. Several mechanisms could be involved in this AR activation [21–23]. Changes in the specificity of AR broadening the responsive spectrum to other steroid hormones different to dihydrotestosterone, can be caused by genetic mutations affecting different regions of AR. We studied if active Stat3 could be sufficient to alter the specificity of a wild-type AR to non-steroid hormones. Our results indicate that native AR, with no mutations, is responsible only to androgens, being Stat3 not sufficient to alter this feature of AR. Activated Stat3 d oes not affect the specificity of AR to bind to other HREs different form ARE. Other altered response of mutated AR is the paradoxical agonistic effect of antiandrogens. Also in this case, Stat3 could not reverse the antagonistic activity of flutamide on a wild-type AR. These results point to mutations in AR as a necessary step in some of the altered responses of AR seen in advanced prostate cancer.

Another possible mechanism by which a prostate cancer circumvents the low levels of androgens resulting from androgen ablation therapy is by increasing the sensitivity of AR to very low levels of androgens [42]. Stat3 increased the sensitivity of AR to a dose of androgen that showed no activity in the absence of Stat3, in the absence of a mutated AR. The constitutive activation of Stat3 found in prostate cancer [27,43] could be an initial step in the clonal selection of malignant cells affecting not only Stat3-regulated genes but also AR, as part of the transcription complex recruited in response to activated AR. Recently, overexpression of SRC-1 and TIF-2 has been found in prostate cancer recurrence after androgen deprivation therapy [44], providing a molecular basis for AR activation that could be similar to the one displayed by Stat3 in its role as coactivator.

In conclusions, we report here that: 1) a constitutively active form of Stat3 stimulates transcriptional activity of SHRs in a hormone-dependent manner; 2) active Stat3 can act synergistically with other c oactivators to stimulate SHRs transcriptional activity; 3) Stat3 did not affect specificity of AR to other steroid hormones different than androgen, or binding of AR to other HREs; 4) Stat3 increases the sensitivity of AR to androgen. These results can help to elucidate the mechanism by which SHRs in general, and AR in particular, regulate gene expression.

Methods

Plasmids

Luciferase reporter plasmids containing specific responsive elements for AR, GR, and PR (GRE-TATA-luciferase) and ER (ERE-TATA-luciferase) have been described elsewhere [29,30]; another androgen-responsive reporter contained the fragment -286/+28 of the rat Probasin (PB)

promoter, and was obtained from Dr. R. Matusik, Vanderbilt University, TN [31]. An expression plasmid for wild-type hAR was kindly provided by Dr. C. Chang, University of Rochester, NY. Expression plasmids for hGR, hPR α and ER have been also described [29,30]. Wild-type inactive form of Stat3 (pCAGGS-HA-Stat3) was from Dr. T. Hirano, Osaka University, Japan [32]. The plasmid expressing a constitutively active form of Stat3 (Stat3C-Flag) was obtained from Dr. J. Darnell, jr. [18]. This constitutively activated Stat3 (a mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3) induces cellular transformation and tumor formation in nude mice [18]. The plasmids to express other coactivators (SRC-1, CBP/p300, TIF2 and PCAF) have been described elsewhere [29,30].

Cells and transfections

CV-1 cells were maintained in 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD), 100 u/ml penicillin, 100 μg/ml streptomycin in DMEM. Twenty four hours before transfection, cells were plated in 12 well plates (1.2 × 105 per well) in 5% charcoal stripped serum (Hyclone, Logan, UT), antibiotics, and 2 mM L-glutamine in phenol red-free DMEM. For transfections, we used (per well) 0.2 μg reporter, 0.2 μg receptor, and 2 μg coactivator or empty vector. In experiments studying synergy of coactivators, the total amount of DNA was kept constant at 4.4 μg per well. Transfections were performed with Superfect reagent according to the manufacturer's protocol (Qiagen, Valencia, CA). Three hours after transfection, medium was removed and hormones added in phenol red-free DMEM containing 5% charcoal stripped serum and antibiotics. Dihydrotestosterone (DHT), dexamethasone (Dex), estradiol (E_2), and the synthetic anti-androgen flutamide were from Sigma (Saint Louis, MO). The synthetic analogs of androgen and progesterone, R1881 and R5020, respectively, were from New England Nuclear (Boston, MA).

Luciferase activity

Thirty six hours after incubation with hormones, luciferase activity was determined in cell extracts according to manufacturer's suggestions (Promega, Madison, WI). Luciferase activity was normalized per μg protein, determined by Bradford assay (Coomasie Plus, Pierce, Rockford, IL). Data are presented as a representative experiment, which was independently repeated at least three times.

Authors' Contributions

DeMiguel and Lee carried out the entire experiments. Onate and Gao conceived of the study, and participated in its design and coordination.

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Interleukin-6 protects LNCaP cells from apoptosis induced by androgen deprivation through the Stat3 pathway[1]

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Key words: IL-6, prostate cancer, apoptosis, Stat3, androgen-independence

Running title: IL-6 confers resistance to apoptosis upon androgen deprivation

Abstracts

Androgen ablation induces apoptotic death of prostate epithelial cells and is a standard treatment for prostate cancer. However, androgen-independent prostate cancer cells become resistant to apoptosis, rendering androgen ablation therapy ineffective. The molecular mechanisms underlying this apoptotic resistance are unclear. Elevated expression of interleukin-6 (IL-6) is implicated in the progression of hormone refractory prostate cancer. Previous studies demonstrated that IL-6 can promote androgen-independent growth of prostate cancer cells. We now provide evidence that overexpression of IL-6 renders androgen sensitive LNCaP human prostate cancer cells more resistant to apoptosis induced by androgen deprivation. LNCaP cells undergo apoptosis after 72 h of androgen deprivation, an outcome is largely absent in clones overexpressing IL-6 as measured by cell death ELISA and chromatin degradation assays. IL-6 over-expressing cells resulted in a significant decrease in the expression of pro-apoptotic proteins such as cleaved PARP and cleaved caspase-9 as well as an increase in the expression of anti-apoptotic proteins Bcl-x_L and phosphorylated Bad. Addition of IL-6 antibody completely abolished the anti-apoptotic activity of IL-6. This protective effect of IL-6 was reversed by the expression of a dominant-negative Stat3 mutant, Stat3F. Furthermore, ectopic expression of a constitutively active Stat3 antagonized androgen deprivation-induced cell death of LNCaP cells. These results indicate that IL-6 protects androgen sensitive LNCaP cells from apoptosis induced by androgen deprivation, and Stat3 activation play an important role in IL-6-mediated anti-apoptosis in prostate cancer cells.

RNA interference targeting Stat3 inhibits growth and induces apoptosis of human prostate cancer cells[1]

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Key words: Stat3, RNA interference, prostate cancer, apoptosis

Running title: Targeting Stat3 by RNA interference in prostate cancer

Abstract

Stat3, a member of the signal transduction and activation of transcription (STAT) family, is a key signal transduction protein that mediates signaling by cytokines, peptide growth factors, and oncoproteins and is constitutively activated in numerous cancers including prostate. Previous studies demonstrated that constitutively activated Stat3 plays an important role in the development and progression of prostate cancer by promoting cell proliferation and protecting against apoptosis. The present study was designed to investigate the potential use of RNA interference to block Stat3 expression and activation and the effect on the growth of human prostate cancer cells. We identified a small interfering RNA (siRNA) specific for Stat3 and expressed in human prostate cancer cells from DNA expression vector which employ RNA polymerase III promoters from the U6 small nuclear RNA gene to transcribe siRNAs. We demonstrate that blockade of Stat3 activation by the Stat3 siRNA suppresses the growth of human prostate cancer cells and Stat3-mediated gene expression and induces apoptotic cell death. The Stat3 siRNA does not inhibit the proliferation nor induces apoptosis of Stat3-inactive human prostate cancer cells. In addition, the Stat3 siRNA inhibits the levels of androgen-regulated prostate specific antigen (PSA) expression in prostate cancer cells. These results demonstrate that targeting Stat3 signaling using siRNA technique may serve as a novel therapeutic strategy for treatment of prostate cancer expressing constitutively activated Stat3.